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Studies have been done in three general areas: i) study of the mechanism of action of botulinum neurotoxin, ii) identification of drugs that antagonize botulinum neurotoxin action, and iii) extension of basic science studies on toxin action to the human nervous system. The two principal tissue preparations used were the mouse phrenic nerve-hemidiaphragm and the human pyramidalis muscle. The major findings were: i) stimulation of transglutaminase activity is not likely to be relevant to clostridial toxin action, ii) zinc chelators, such as TPEN, are intracellular antagonists of all clostridial neurotoxins, iii) inihibitors of vascuolar adenosine triphosphatase, such as bafilomycin, antagonize all clostridial neurotoxins but not phospholipase A2 neurotoxins, and iv) human neuromuscular junctions are sensitive to all botulinum serotypes associated with natural poisoning (e.g., A, B, and E), and they are also sensitive to one serotype that is not typically associated with natural poisoning (e.g., C).

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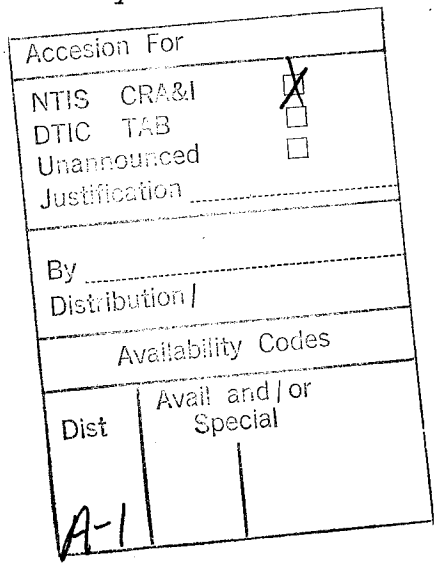

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V. INTRODUCTION

Botulinum neurotoxin acts selectively on peripheral cholinergic nerve endings to block the release of acetylcholine. Motor nerve endings are more sensitive that preganglionic or postganglionic nerve endings of the autonomic nervous system, and thus the major outcome of toxin action is blockade of neuromuscular transmission. In the extreme, toxin-induced blockade can produce complete flaccid paralysis.

A general model has emerged to explain the actions of botulinum neurotoxin on sensitive nerve endings (Simpson, 1986; Simpson, 1989). As originally envisioned, the model proposed three sequential steps [i.e., binding, internalization, and intracellular action; (Simpson, 1980)]. However, it is useful to divide the internalization step into two separate events (e.g., penetration of the plasma membrane and penetration of the endosome membrane). This is particularly helpful in the context of efforts to develop pharmacological antagonists of toxin action. Therefore, botulinum neurotoxin action will be described as a series of four events.

Binding. Botulinum toxin binds selectively to motor nerves, and even more precisely to those portions of the presynaptic membrane that are juxtaposed to endplate regions (Black and Dolly, 1986a; Black and Dolly, 1986b). The toxin does not bind to muscle, to myelinated or demyelinated segments of axon, or to tissues remote from the nerve terminal.

The receptor for botulinum neurotoxin has not been isolated and characterized, but there are at least three features of the receptor that have been established. First, the binding of botulinum neurotoxin to the plasma membrane does not alter neuromuscular transmission (Burgen et al., 1949; Simpson, 1980). Thus, it is unlikely that the exposed portion of the receptor plays a critical role in junctional transmission. Second, there is strong evidence that more than one receptor is implicated in neurotoxin action. Botulinum neurotoxin exists in seven serotypes designated A, B, C, D, E, F and G. These several serotypes do not share a common receptor, and thus there must be multiple receptors that mediate toxin binding (Habermann and Dreyer, 1986; Middlebrook, 1989). Third, there is highly suggestive evidence that a sialic acid-containing molecule plays a role in toxin binding (Simpson and Rapport, 1971a; Simpson and Rapport, 1971b; Bakry et al., 1991a). One possible explanation is that a ganglioside acts cooperatively with another molecule

such as a protein to create a binding site (Montecucco, 1986). Another possibility is that the receptor is a sialoglycoprotein (Schiavo et al., 1991).

Endocytosis. Botulinum neurotoxin crosses the plasma membrane by the process of receptor-mediated endocytosis, and this process is blocked by low temperature and by drugs that poison cellular metabolism (Black and Dolly, 1986a; Black and Dolly, 1986b). The fact that botulinum neurotoxin acts locally at the nerve terminal suggests that it is internalized by a population of endosomes that remains in the nerve ending (viz., endosomes that meld with lysosomes) rather than a population that undergoes retrograde axonal transport to the cell body (viz., endosomes that transport tetanus toxin and various virus particles).

Translocation. Botulinum neurotoxin must escape the endosomal lumen and reach the cytosol to exert its poisoning effects. There is some agreement about initiation of this event, but there is uncertainty about how it is completed. Most investigators agree that translocation is an acid-dependent event (for review, see Simpson, 1993a). As the proton pump in the endosome membrane progressively lowers intraluminal pH, the toxin molecule undergoes a conformational change. The most important aspect of this conformational change is exposure of an occult hydrophobic domain. This newly exposed hydrophobic region inserts into the endosome membrane, thus triggering translocation, but it is unclear how translocation is actually achieved. Various possibilities are: *i.)* a portion of the molecule may serve as a tunnel protein to accommodate passage of the poisoning domain, *ii.)* a portion of the molecule may serve as a leader sequence to achieve translocation, *iii)* several hydrophobic domains may act in concert to achieve a form of bulk transfer, or *iv.)* the molecule could lyse the endosome membrane. There is as yet no compelling evidence in favor of any particular model.

Intracellular poisoning. In the recent past there has been a surge of published work dealing with the intracellular effects of clostridial neurotoxins. Three hypotheses have emerged as proposed explanations for toxin action: *i.)* inhibition of intracellular fusogens, *ii.)* stimulation of transglutaminase and intracellular cross-linking, and *iii.)* proteolytic cleavage of peptides that mediate membrane fusion.

During the contract period, the principal investigator conducted a series of studies that are

linked to the proposed model for toxin action. First, work was done to show that transglutaminase activity is not likely to be relevant to the mechanism of clostridial toxin action. Next, experiments were done to show that inhibitors of vacuolar ATPase antagonize productive internalization of toxin, and zinc chelators antagonize the intracellular actions of toxin. Finally, work was done to extend several basic science findings on toxin action to the human nervous system.

VI. GENERAL METHODS AND TECHNIQUES

Toxins and drugs. Botulinum neurotoxin type B was kindly provided by Dr. Y. Kamata (University of Osaka Prefecture), and botulinum neurotoxin type G was kindly provided by Dr. M. Nukina (Public Health Research Institute of Kobe City). Botulinum neurotoxin type A was isolated as previously described (Simpson et al., 1988). Serotypes C, D, E and F were purchased from WAKO Fine Chemicals. Tetanus toxin was purchased from Calbiochem.

Botulinum neurotoxin types B and E were converted from the unactivated to the activated forms by incubation with N-tosyl-phenylalanine chloromethylketone-treated trypsin that was coupled to agarose beads [trypsin:toxin; 1:40 (W:W)]. The mixture was incubated for 30 min at 35° C, and the reaction was terminated by centrifugation and subsequent aspiration of activated toxin. The homogeneity and molecular structure of the toxins were confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, and the biological activity of the toxins was assayed on phrenic nerve-hemidiaphragm preparations.

EDTA, DTPA and TPEN were purchased from Molecular Probes; *Triticum vulgaris* lectin was obtained from Sigma Chemical Company; all salts and reagents were from Sigma Chemical Company or Fisher Scientific Company.

Neuromuscular preparations. Mouse phrenic nerve hemidiaphragm preparations were excised and used to monitor stimulus-evoked muscle twitch, stimulus-evoked endplate potentials, and spontaneous endplate potentials. In all cases, tissues were suspended in a physiological solution of the following composition (mM): NaCl, 137; KCl, 5; $CaCl_2$, 1.8; $MgSO_4$, 1.0; $NaHCO_3$, 24; Na2HPO_4, 1.0; and d-glucose, 11. The solution was augmented with gelatin (0.01 - 0.02%) as an auxiliary protein to diminish non-specific adsorption or inactivation of toxin.

During experiments on evoked twitch, tissues were suspended in a 25 ml bath. Phrenic nerves were stimulated with bipolar electrodes at 0.3 Hz, and muscle responses were monitored with a strain gauge transducer and physiological recorder. Toxin-induced paralysis was measured as a 90% reduction in twitch response to nerve stimulation. During experiments on endplate

responses, tissues were pinned in a small petri dish (< 5 ml) and continuously perfused (1 ml/min) with fresh physiological solution (34° C) of the composition given above. The phrenic nerve was stimulated intermittently with a suction electrode filled with 3 M KCl and a current pulse of 1 ms. Standard techniques were used to obtain recordings with glass microelectrodes filled with 3M KCl (tip resistance, 20 to 40 MΩ). Resting membrane potentials ranged from -60 to -80 mV.

MEPP's were recorded with a high input impedance amplifier. The output from the amplifier was further amplified, filtered at 5 kHz by low pass filter, and digitized through an A/D converter interfaced with a computer. Data were stored and later analyzed with Axotape and Pclamp software. Separation of MEPP activity from background was achieved with a window discriminator in the software. In general, a minimum of 10 endplates were sampled per tissue. MEPP's were recorded for a period of 1 to 2 minutes per endplate, and the average MEPP frequencies for the endplates in each muscle were determined.

During control experiments on MEPP's, tissues were incubated in bathing medium at 34° C and endplate activity was measured for a baseline period of 30 to 60 min prior to addition of toxin. During experiments with zinc chelators, tissues were pretreated with drugs for 120 min at 34° C. Baseline activity was recorded for 30 to 60 min prior to addition of toxin. Depending on experimental conditions, the frequency of spontaneous MEPP's was recorded for 70 to 140 min after addition of toxin.

In most experiments toxins were added to baths at 34° to 35° C and phrenic nerves were stimulated as described above. Mechanical or electrophysiological responses were monitored until tissues became paralyzed. The one exception to this general rule pertained to studies on internalization of toxin. In this situation, toxins were added to tissues at 4° C and phrenic nerves were not stimulated. Incubation was continued for 60 min, which has been shown to be adequate for binding (Simpson, 1980). Tissues were then washed to remove unbound toxin, temperature was raised to 35° C, and phrenic nerves were stimulated. Raising temperature to physiological levels and applying nerve stimulation initiated internalization of membrane-bound toxin.

The rate of internalization of toxin was quantified by using neutralizing polyclonal antibody as a research tool. At various times after initiation of internalization, a large excess of neutralizing antibody was added to tissue baths. Paralysis times were monitored as a function of the interval between initiation of internalization and addition of antibody.

Binding studies. Proteins were iodinated with Bolton-Hunter reagent according to standard methods. Clostridial neurotoxins (150 μg) in sodium borate buffer (100 mM, pH 7.9) were mixed with ^{125}I-Bolton-Hunter reagent (1 mCi) for 30 minutes at room temperature (final volume, 1 ml). The reaction was terminated by adding 200 mM glycine. The radioiodinated protein was separated from reactants on a Sephadex G-50 column. Protein concentration was determined by the methods of Lowry et al. (1951) or Bradford (1976), and toxicity was bioassayed by the method of Kondo et al. (1984). Residual toxicity of iodinated preparations was 65 to 85 percent.

Brain membrane preparations were obtained from adult Sprague-Dawley rats. Dissected brains were washed and homogenized in iced Tris-HCl buffer (50 mM, pH 7.4), then centrifuged for 10 min at 1000 x *g*. The resulting supernatant was recentrifuged for 45 min at 40,000 x *g*. The pellets were re-suspended in Tris-HCl buffer (as above).

Binding of toxins to brain membrane preparations was measured by a centrifugation assay. Labelled ligand (0.5 nM) was mixed with 200 μg of membrane protein in 1.0 ml of pH 7.4 buffer containing 50 mM Tris-HCl, 100 mM sodium chloride, and 1 mg/ml BSA. The binding reaction was done at 22° C for 90 min, which is the amount of time necessary to reach equilibrium (Bakry et al., 1991a,b). The reaction was terminated by centrifugation (15,000 x *g*, two min), after which the pellet was washed and recentrifuged in fresh buffer. The bottom of the microtube was cut, and the amount of ^{125}I-ligand was quantified. Data were corrected for non-specific binding.

Polyacrylamide gel electrophoresis. The conversion of toxins from the unactivated to the activated forms was monitored by doing polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate as described by Laemmli (1970). Activation involved trypsin-induced nicking of the toxin, during which the single chain molecule (~150,000 daltons) was converted to

a dichain molecule with a heavy chain (~100,000 daltons) linked by a disulfide bond to a light chain (~50,000 daltons). Both the unactivated and the activated forms of the toxins were exposed to reducing conditions (dithiothreitol or mercaptoethanol) before electrophoresis.

VII. STUDIES ON ZINC CHELATION

A. RESULTS

Chelation of metals. Two paradigms were used to test the hypothesis that chelation of metals would antagonize the neuromuscular blocking properties of clostridial toxins. In the first, chelators were incubated with toxin for 120 min at 30° C, after which free chelator and chelator~metal complex were separated from toxin by filtration and centrifugation (molecular weight cutoff = 30,000). The toxin was then added to neuromuscular preparations, and paralysis times were monitored. In the second paradigm, chelators were added to tissues for 120 min at 35° C. Untreated toxin was then added to tissues, and paralysis times were monitored. Experiments were done with botulinum neurotoxin type A and type B, which are prototypes of the two known classes of clostridial neurotoxins (see Comment).

Experiments involving pretreatment of toxin with chelators were uniformly negative. When tested individually or in combination at 200 μM, EDTA, DTPA and TPEN did not have a significant effect on toxin-induced neuromuscular blockade. This outcome was obtained when the toxin:chelator ratio was 1:10,000 or greater. Even when tested at 10 mM, EDTA did not alter the biological activity of botulinum neurotoxin type A (1×10^{-11} M) or type B (3×10^{-11} M).

Experiments involving pretreatment of tissues produced an outcome that was dependent on the chelator under study. When tested at concentrations of 40 μM to 160 μM, EDTA had only a modest effect on the potency of botulinum neurotoxin. However, DTPA and especially TPEN produced concentration-dependent antagonism of both botulinum neurotoxin type A and type B (Fig. 1). The apparent EC50 for DTPA was in the range of 20 to 40 μM. The apparent EC50 for TPEN was in the same range, although the exact value was difficult to quantify. TPEN was so effective in producing antagonism that the ability to monitor toxin-induced blockade was hampered by spontaneous deterioration of tissues. Phrenic nerve-hemidiaphragm preparations began to show significant decay when studied for periods greater than 6 to 7 hours. In any event, the antagonism produced by TPEN was substantial.

The paradigm of pretreating tissues with chelators was extended to include all serotypes of botulinum neurotoxin and tetanus toxin. The pretreatment (120 min; 35° C) involved a combination of DTPA and TPEN (each at 30 μM); the toxins were tested individually at approximately equiactive concentrations. The results showed that the chelators antagonized every clostridial neurotoxin (Table 1).

The data illustrated in Table 1 for botulinum neurotoxin type B and type E pertain to activated species. Identical experiments were done with unactivated type B, which possessed only 1 to 2% of the potency of activated type B. There was no significant difference in the amount of protection provided by a combination of DTPA and TPEN when tested against equipotent concentrations of unactivated (0.5×10^{-9} M) and activated (1×10^{-11} M) type B toxin (not illustrated).

Reversal of antagonism. Two approaches were used to demonstrate reversibility of the antagonism produced by TPEN: washing chelator from tissues or adding an excess of zinc. Tissues were divided into three matched groups (group n = 3 or more), as follows: *i.)* tissues that were neither pretreated with chelator nor incubated in chelator during exposure to toxin (i.e., control), versus tissues that were both pretreated with chelator (120 min) as well as treated with chelator during exposure to toxin, *ii.)* control tissues, versus tissues that were pretreated with chelator but then washed to remove chelator during exposure to toxin, and *iii.)* control tissues, versus tissues that were pretreated with chelator and then treated both with chelator and with an excess of zinc (125 μM) during exposure to toxin. In all cases, TPEN was used at a concentration of 30 μM.

Experiments were done with botulinum neurotoxin type A and type B, and the results with the two serotypes were closely similar. The data for serotype A are illustrated in Figure 2. As expected, pretreatment of tissues with a zinc chelator and continued treatment with chelator during exposure to toxin produced highly significant antagonism ($p < 0.001$). Tissues that were pretreated with chelator, but then washed prior to addition of toxin, survived significantly longer than control tissues ($p < 0.01$) but significantly shorter than tissues exposed to chelator

throughout the experiment ($p < 0.01$). Tissues that were pretreated with chelator and then treated with chelator plus an excess of zinc during exposure to toxin did not differ significantly from control tissues ($p > 0.05$).

Specificity of antagonism. The two paradigms used to test the hypothesis that chelation of zinc would antagonize clostridial neurotoxins were also used to determine whether chelation would antagonize phospholipase A2 neurotoxins. The experimental protocols were identical to those described above; the phospholipase A2 neurotoxins studied were notexin (1×10^{-8} M), crotoxin (1×10^{-7} M), and β-bungarotoxin (1×10^{-8} M). In every case the results were negative (not illustrated). Pretreatment of toxins with EDTA, DTPA and TPEN, each at 200 μM (100-fold or greater molar excess relative to toxin), did not alter the apparent potency of notexin, crotoxin or β-bungarotoxin (i.e., $p > 0.05$). Similarly, pretreatment of tissues with chelators, again at 200 μM, did not delay the onset of phospholipase A2 toxin-induced neuromuscular blockade ($p > 0.05$).

Analysis of binding. The binding of botulinum neurotoxin type A and type B to rat brain membrane preparations was studied as previously described (Bakry et al., 1991a,b). Binding of the toxins demonstrated specificity and saturability, as previously reported (Bakry et al., 1991a,b).

Three classes of toxin antagonists were tested for their abilities to alter binding: *i.)* homologous unlabeled toxin, *ii.)* a lectin that has been shown to antagonize the binding of all clostridial neurotoxins (*Triticum vulgaris* lectin; 30 μM; Bakry et al., 1991a), and *iii.)* two chelators that were shown above to antagonize neuromuscular blockade by clostridial neurotoxins (DTPA and TPEN, each at 200 μM).

The results confirmed that homologous toxin as well as lectin with affinity for sialic acid antagonize the binding of botulinum neurotoxin type A (Fig. 3A) and type B (Fig. 3B). However, neither DTPA nor TPEN affected the binding of either toxin.

Analysis of internalization. Phrenic nerve-hemidiaphragm preparations were incubated with botulinum neurotoxin type A (3×10^{-11} M) or type B (1×10^{-10} M) for 60 min at 4° C, after

which they were washed to remove unbound toxin. Tissues were transferred to baths at 35° C and nerve stimulation was applied. At various times thereafter, neutralizing antibodies were added and paralysis times were monitored.

The experimental protocol with antibody was done on two sets of tissues: a control set that was not exposed to TPEN or zinc, and an experimental set that was pretreated for 120 min with TPEN (20 μM) and then treated with zinc (120 μM). In the latter case, zinc was added to tissues at the same time as antibody.

The results with botulinum neurotoxin type A and type B were qualitatively the same (Fig. 4). When antibody was added immediately to control tissues, it provided substantial protection against toxins. As the interval between raising temperature and adding antibody increased, the magnitude of protection diminished. When the interval was 30 min or longer, there was no significant protection. Interestingly, the magnitude and temporal characteristics of the antibody effect in control tissues were not significantly different from those observed in tissues pretreated with TPEN.

Analysis of intracellular action. The rate of spontaneous MEPP's was measured in control tissues exposed to botulinum neurotoxin type A (1×10^{-10} M) and in experimental tissues pretreated with a zinc chelator (120 min) and then exposed to toxin (Figure 5). The mean baseline frequency of MEPP's in control tissues was 1.5 per sec ($n = 4$). Following addition of toxin, MEPP frequency fell to approximately 50% of baseline activity within 35 min, and activity further declined to 3% within 65 min. The mean baseline frequency of MEPP's in tissues pretreated with DTPA (40 μM) was 1.7 per sec ($n = 3$). This value is not significantly different from that observed in control tissues. Following addition of toxin, MEPP frequency fell to approximately 50% of baseline within 65 min, and it fell to 3% of baseline in approximately 120 min. The mean baseline frequency of MEPP's in tissues pretreated with TPEN (20 μM) was 1.3 per sec ($n = 3$), a value that is not significantly different from that seen in control tissues. However, the chelator provided substantial protection against the toxin. The spontaneous MEPP frequency in tissues pretreated with TPEN did not decrease 50% within the 140 min observation period.

Combinations of antagonists. Tissues were suspended in physiological solution for 30 min at 4° C in the absence or presence of lectin from *Triticum vulgaris* (1×10^{-4} M). Tissues were suspended for an additional 60 min at 4° C in the presence of botulinum neurotoxin type A (3×10^{-11} M) or type B (1×10^{-10} M). Following these procedures, tissues were washed and transferred to baths at 35° C. Phrenic nerves were stimulated, and paralysis times were monitored. As expected (Bakry et al., 1991a), the lectin provided significant protection against the toxins (Table 2).

A similar protocol was used in the next series of experiments, except that tissues were pretreated with TPEN (30 μM; 60 min, 35° C) and the chelator was present during all subsequent steps (incubation with lectin/toxin at 4° C; stimulation at 35° C). The results showed that tissues pretreated with TPEN were significantly protected, and those pretreated with TPEN and later incubated with lectin were even more protected (Table 2).

Combination experiments were also done with methylamine hydrochloride. Tissues were incubated in the absence or presence of drug (15 mM) for 60 min at 35° C. Botulinum neurotoxin type A (1×10^{-11} M) or type B (3×10^{-11} M) was then added to tissues, phrenic nerves were stimulated, and paralysis times were monitored. In keeping with previous findings (Simpson, 1983), the drug afforded substantial protection (Table 2).

In companion studies, tissues were pretreated with TPEN (30 μM; 60 min, 35° C), and the chelator was present during subsequent steps (incubation with methylamine and exposure to toxin). Once again, the results showed that the chelator provided significant protection against botulinum neurotoxin, and the combination of chelator plus methylamine hydrochloride provided even greater protection (Table 2).

B. COMMENTS

Botulinum neurotoxin and tetanus toxin are synthesized as single chain molecules with an Mr of approximately 150,000. These relatively inactive precursors undergo proteolytic processing to

give a dichain molecule in which a heavy chain (Mr ~ 100,000) is linked by a disulfide bond to a light chain (Mr ~ 50,000). These dichain molecules act on vulnerable cells to block release of mediators.

There is a sequence of steps through which the toxins pass to exert their effects (see review by Simpson, 1993). These include a binding step at the plasma membrane, a receptor-mediated internalization step to penetrate the plasma membrane, an acid-dependent translocation step to penetrate the endosome membrane, and an intracellular poisoning step. A complete structure-function analysis of this sequence has not been determined, but two major points have been established. The heavy chain possesses the tissue-targeting domain that directs the toxins to cells (Goldberg et al., 1981; Bandyopadhyay et al., 1987), and the light chain acts in the cytosol to block exocytosis (Bittner et al., 1989a,b; Ahnert-Hilger et al., 1989).

It has long been assumed that the intracellular poisoning step is enzymatic in nature, and recent work provides strong support for that belief. Clostridial neurotoxins have a histidine motif that is characteristic of zinc metalloendoproteases, and this characteristic sequence is located in the light chain components of the toxins. Furthermore, the light chains of the toxins have been shown to be zinc binding proteins, and all of the toxins express zinc-dependent enzymatic actions. Botulinum neurotoxins are endoproteases that cleave peptides necessary for exocytosis (Schiavo et al., 1992a; Schiavo et al., 1993; Link et al., 1992).

The evidence available suggests that the toxins express a zinc-dependent action, and perhaps a zinc-dependent endoprotease action. This leads to the hypothesis that zinc-chelators, and perhaps specific inhibitors of metalloendoproteases, may be toxin antagonists. The present study has addressed the possibility that zinc chelators are universal antagonists of clostridial neurotoxin action at the mammalian neuromuscular junction.

Antagonistic effects of EDTA, DTPA and TPEN. Clostridial neurotoxins are typically divided into two classes based on their interactions with pharmacological agents (Sellin et al., 1983a,b; Kauffman et al., 1985; Dreyer et al., 1987; Gansel et al., 1987). Botulinum neurotoxin

type A is a prototype of one class, and botulinum neurotoxin type B and tetanus toxin are prototypes of the second class. Chelators can also be divided into different classes, and for the purpose of this report it is convenient to classify them based on their relative affinities for calcium and zinc. EDTA is a broad spectrum agent that is particularly useful for chelating calcium, DTPA is somewhat intermediate, and TPEN is an agent with greater relative affinity for zinc. These three chelators have been studied as potential antagonists of prototype clostridial neurotoxins, and in some cases as antagonists of all clostridial neurotoxins.

EDTA, DTPA and TPEN produced antagonism of the neuromuscular blocking properties of botulinum neurotoxin type A and type B, but the magnitude of antagonism depended both on the nature of the experimental conditions and on the chelator. Pretreatment of toxins with a large molar excess of the chelators did not produce any reduction in potency. This result was obtained even when the ratio of chelator to toxin was more than adequate to remove zinc (Schiavo et al., 1992b). The likely explanation for this outcome is that tissues possess sufficient stores of zinc to allow metal rebinding by the toxin and thus restoration of biological activity. However, when tissues were pretreated with chelator and then exposed to toxin, there was a concentration-dependent antagonism of toxin-induced neuromuscular blockade.

It should be noted that pretreatment of tissues with chelators most likely had two effects. The extracellular chelator could remove zinc from toxin, and the intracellular chelator could remove free zinc from the cytosol and prevent metal rebinding by the toxin. It seems reasonable to assume that both of these effects contributed to the observed antagonism.

Because of the marked effect seen when chelators were tested against botulinum neurotoxin type A and type B, DTPA and TPEN were tested against the six remaining clostridial neurotoxins. The results demonstrated that chelators were antagonists of every serotype of botulinum neurotoxin as well as tetanus toxin. This outcome qualifies chelators of zinc to be regarded as universal antagonists of clostridial neurotoxins (and see below).

Site of antagonism. Experiments were done to assure that chelators were acting to

antagonize intracellular expression of toxicity rather than some other step in the progression of clostridial neurotoxin action. This work demonstrated that the chelators were not antagonists of binding nor were they antagonists of productive internalization. Furthermore, the chelators were not effective in promoting acetylcholine release, which means they could not have been acting indirectly to delay toxin action.

Experiments on binding involved iodinated toxin and nerve membrane preparations, as previously described (Bakry et al., 1991a,b). Binding was studied in the presence of homologous unlabeled toxin, *Triticum vulgaris* lectin, DTPA and TPEN. As expected, homologous toxin and lectin strongly antagonized binding. However, the two chelators, even when tested at substantial concentrations (200 μM), had no effect.

Experiments on internalization utilized a paradigm that was developed to monitor toxin penetration of the plasma membrane (Simpson, 1980; Simpson and DasGupta, 1983). When clostridial neurotoxins are incubated with tissues at low temperature and in the absence of nerve stimulation, they bind tightly to membranes but at the same time they remain accessible to the neutralizing effects of polyclonal antibody. When temperature is raised and nerve stimulation is applied, the toxins penetrate the plasma membrane by receptor-mediated endocytosis. Toxin that has crossed the membrane is no longer accessible to extracellular antibody. Thus, the rate at which bound toxin disappears from accessibility to extracellular antibody is a measure of the rate of internalization.

The rate at which botulinum neurotoxin type A and type B crossed the plasma membrane was studied in the presence and absence of TPEN. To ensure that the chelator was not acting at any step beyond internalization, an excess of zinc was added simultaneously with polyclonal antibody. Control experiments showed that toxin penetrated the plasma membrane within approximately 30 min, which is closely in keeping with prior results (Simpson, 1980; Simpson and DasGupta, 1983). The same outcome was obtained in the presence of TPEN, making it unlikely that this agent alters the rate or extent of toxin penetration of the plasma membrane.

The next step in toxin action is penetration of the endosome membrane. This involves a pH-dependent change in toxin conformation that allows an occult hydrophobic domain to insert into the membrane and initiate translocation to the cytosol. In preliminary experiments, the authors have found that chelation of zinc does not alter the characteristic pH-induced change in toxin conformation (unpublished). This work has also shown that chelation does not block pH-induced partitioning of toxin from an aqueous to an organic phase.

The final step in toxin action is the presumed metalloendoprotease cleavage of an intracellular peptide essential for exocytosis. Such an action has been demonstrated for botulinum neurotoxin type B, type F, and tetanus toxin, and a similar action may be shared by the other neurotoxins. Indeed, the observation that chelation antagonizes all clostridial neurotoxins is itself an indication that these agents share a similar zinc-dependent action. This belief is strengthened by the fact that chelators do not exert non-specific effects to promote transmitter release (e.g., increase spontaneous MEPP's). In the absence of an indirect action on nerve endings to enhance exocytosis, the chelators would appear to be acting directly on the toxins to inhibit blockade of exocytosis.

Universal antagonists. Clostridial neurotoxins share many structural and functional properties, but there are also major differences. For example, the several toxins do not share the same receptor; to the contrary, each serotype appears to have its own unique receptor. As another example, the various botulinum neurotoxins cleave different substrates.

The fact that there are differences has prompted an effort to identify universal antagonists. By definition, these are compounds that exert effects on all serotypes of botulinum neurotoxin and tetanus toxin, in spite of the fact that there are inherent differences in the toxins themselves (Simpson, in press). To date, two groups of universal antagonists have been identified. Lectins with affinity for sialic acid, such as those from *Triticum vulgaris* and *Limax flavus*, antagonize the binding of all clostridial neurotoxins (Bakry et al., 1991a). This is due to the fact that the receptors for the toxins - or the immediate microenvironment of these receptors - share the property of an exposed sialic acid residue. In addition, drugs that block (e.g., bafilomycin) or

reverse (e.g., methylamine hydrochloride) acidification of endosomes antagonize all clostridial neurotoxins (Simpson, 1982,1983). This is due to the fact that productive internalization of toxins involves an acid-dependent step that initiates translocation from endosomes to the cytosol.

The present report introduces a third group of universal antagonists. Heavy metal chelators, and especially those with high affinity for zinc, delay the expression of biological activity by all serotypes of botulinum neurotoxin and tetanus toxin. Furthermore, chelators such as TPEN are equivalent to, if not more effective than, any of the previously described groups of antagonists, and they can be used in combination with other antagonists to produce marked reductions in the apparent potency of toxins. These findings encourage efforts to identify the correct combination of antagonists, and an appropriate mechanism for delivery of antagonists, to achieve a beneficial outcome in patients poisoned with botulinum neurotoxin or tetanus toxin.

TABLE 1

Effects of chelators on the neuromuscular blocking properties of clostridial toxins

Toxin[a]	Chelators[b]	Paralysis Time[c]
Serotype A	-	84 ± 6
Serotype A	+	296 ± 24
Serotype B	-	99 ± 8
Serotype B	+	201 ± 13
Serotype C	-	78 ± 5
Serotype C	+	313 ± 29
Serotype D	-	91 ± 8
Serotype D	+	244 ± 19
Serotype E	-	92 ± 7
Serotype E	+	223 ± 13
Serotype F	-	81 ± 9
Serotype F	+	214 ± 17
Serotype G	-	79 ± 8
Serotype G	+	251 ± 26
Tetanus toxin	-	81 ± 4
Tetanus toxin	+	201 ± 11

[a] The seven serotypes of botulinum neurotoxin and tetanus toxin were added individually to tissues at approximately equiactive concentrations. The *n* for each group was 4 or more.

[b] Tissues were exposed to toxin in the absence (-) or presence (+) of chelators. In the latter, tissues were pretreated with a combination of DTPA and TPEN (each at 30 μM; 120 min).

[c] Paralysis times were measured as described under Methods. The results are presented as the mean ± SEM. For each toxin, the paralysis times in the presence of chelators were significantly different from those in the absence of chelators ($p < 0.001$).

TABLE 2

The effects of antagonists on the neuromuscular blocking properties of clostridial toxins

Toxin[a]	Antagonist[b]	Paralysis Times[c]
Serotype A	None	94 ± 5
Serotype A	Lectin	162 ± 14
Serotype A	TPEN	245 ± 13
Serotype A	Lectin + TPEN	331 ± 26
Serotype B	None	86 ± 9
Serotype B	Lectin	142 ± 12
Serotype B	TPEN	199 ± 16
Serotype B	Lectin + TPEN	284 ± 16
Serotype A	None	91 ± 6
Serotype A	Methylamine	170 ± 17
Serotype A	TPEN	225 ± 19
Serotype A	Methylamine + TPEN	313 ± 21
Serotype B	None	90 ± 3
Serotype B	Methylamine	151 ± 12
Serotype B	TPEN	205 ± 19
Serotype B	Methylamine + TPEN	278 ± 20

[a] Botulinum neurotoxin type A was used at a concentration of 3×10^{-11} M during experiments with *Triticum vulgaris* lectin and at a concentration of 1×10^{-11} M during experiments with methylamine hydrochloride. Botulinum neurotoxin type B was used at a concentration of 1×10^{-10} M during lectin experiments and at a concentration of 3×10^{-11} M during methylamine experiments. The *n* for each group was five.

[b] Tissues were exposed to toxin in the absence or presence of antagonists, as indicated. The concentrations of the antagonists were *Triticum vulgaris* lectin, 100 μM; TPEN, 30 μM; and methylamine hydrochloride, 15 mM. Experimental conditions are described in the text.

[c] Paralysis times were measured as described under Methods. The results are presented as the mean ± SEM.

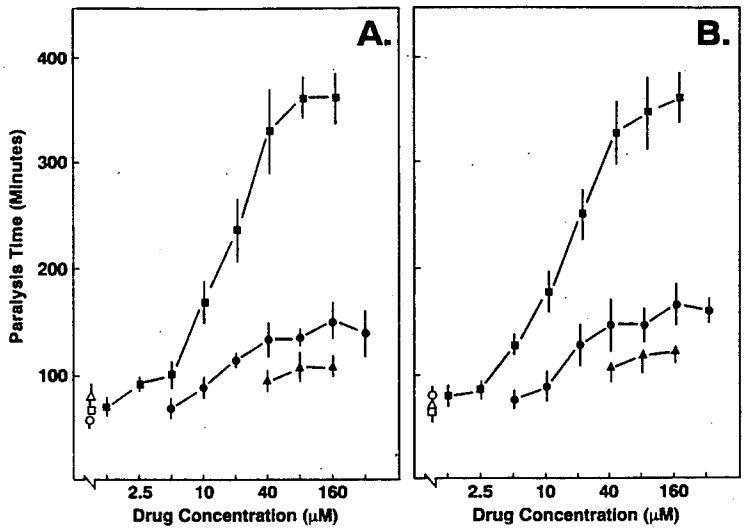


Fig. 1. The effects of various concentrations of EDTA (▲), DTPA (●) and TPEN (■) on the neuromuscular blocking properties of botulinum toxin type A (1×10^{-11} M; A.) and type B (3×10^{-11}; B.). The paralysis times of control tissues (open symbols) were compared with those of experimental tissues (filled symbols) that were pretreated with chelators (120 min). The results indicated that chelators antagonized the neuromuscular blocking properties of toxins, and the rank order of the chelators in producing antagonism was TPEN ≫ DTPA > EDTA.

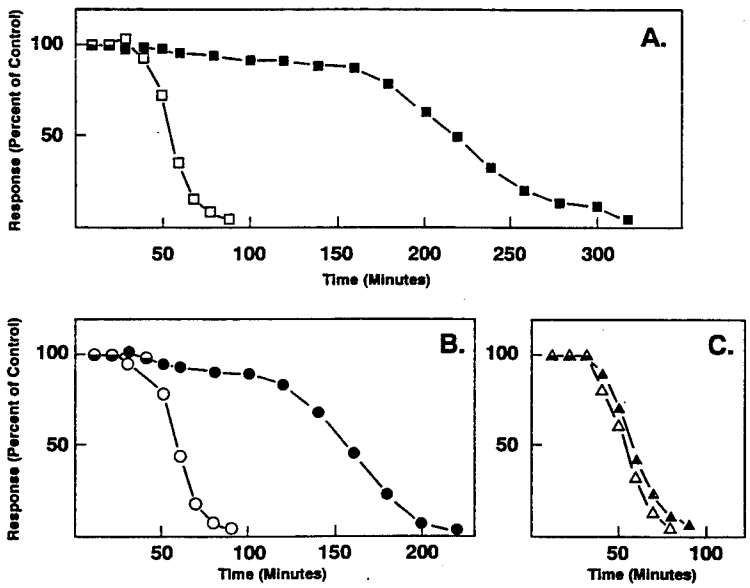


Fig. 2. The effects of TPEN on botulinum toxin type A-induced neuromuscular blockade. Phrenic nerve-hemidiaphragm preparations were divided into three sets of matched groups, as described in the text. In each set, the paralysis times of control tissues (open symbols) were compared with the paralysis times of tissues pretreated with TPEN (filled symbols; 30 μM; 120 min). All tissues were paralyzed with botulinum neurotoxin type A (1×10^{-11} M). Part A of the figure illustrates the magnitude of the effect evoked by pretreating tissues with TPEN. Part B illustrates the effect of pretreating tissues with TPEN and then washing the drug from the tissues immediately before adding toxin. Part C illustrates the effect of pretreating tissues with TPEN and then adding an excess of zinc (125 μM) simultaneously with toxin. Each data point represents the mean value obtained from three or more tissues.

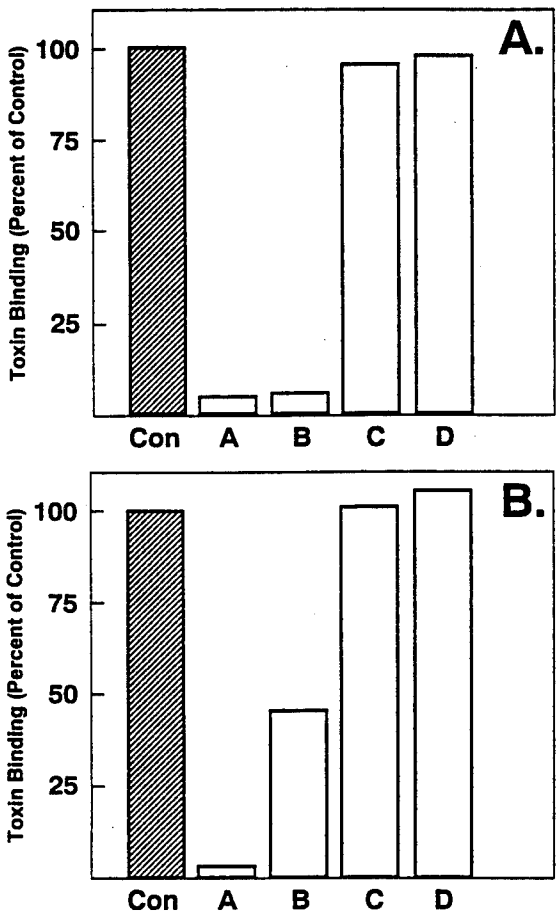


Fig. 3. The binding of iodinated botulinum neurotoxin type A (A.) and B (B.) to nerve membrane preparations. The amount of specific binding (see text for details) was determined for control preparations (Con) as well as for experimental preparations preincubated with homologous, unlabeled toxin (A; 1×10^{-7} M), *Triticum vulgaris* lectin (B; 30 μM), DTPA (C; 200 μM) and TPEN (D; 200 μM). In all cases, labeled toxin was used at a concentration of 5×10^{-10} M. The actual value for specific binding of botulinum neurotoxin type A to control membranes was 592,522 ± 15,627 dpm per 0.1 mg protein; the actual value for specific binding of botulinum neurotoxin type B was 269,316 ± 6,340 dpm per 0.1 mg protein.

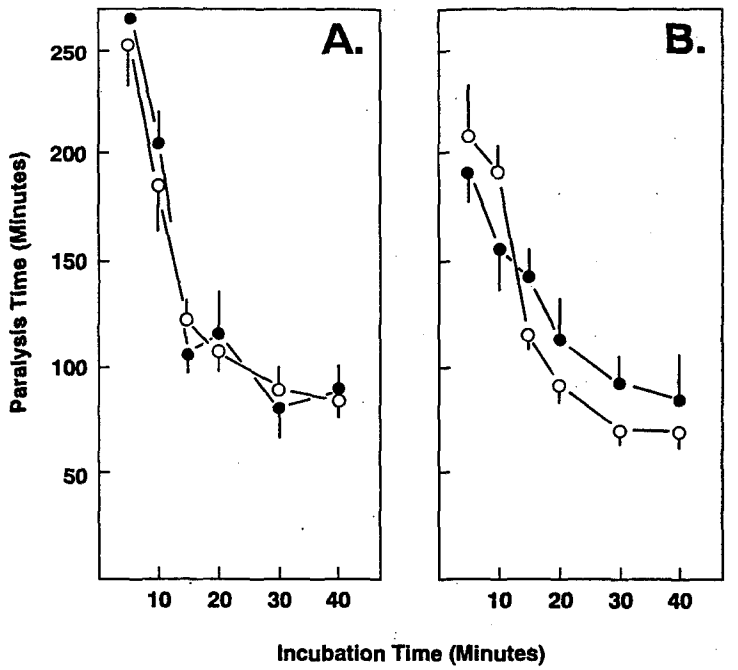


Fig. 4. The effects of neutralizing antibodies on the neuromuscular blocking properties of botulinum toxin type A (1×10^{-11} M; A.) and type B (3×10^{-11} M; B.). Toxin was allowed to bind to tissues as described in the text. Tissues were then washed to remove unbound toxin and transferred to conditions for monitoring the development of paralysis. Antibody was added at various times after transfer, and paralysis times were monitored. The experiment was done both in the absence (open symbols) and in the presence (filled symbols) of TPEN. In the latter case tissues were pretreated with the chelator (20 μM; 120 min), and an excess of zinc (120 μM) was added simultaneously with antibody.

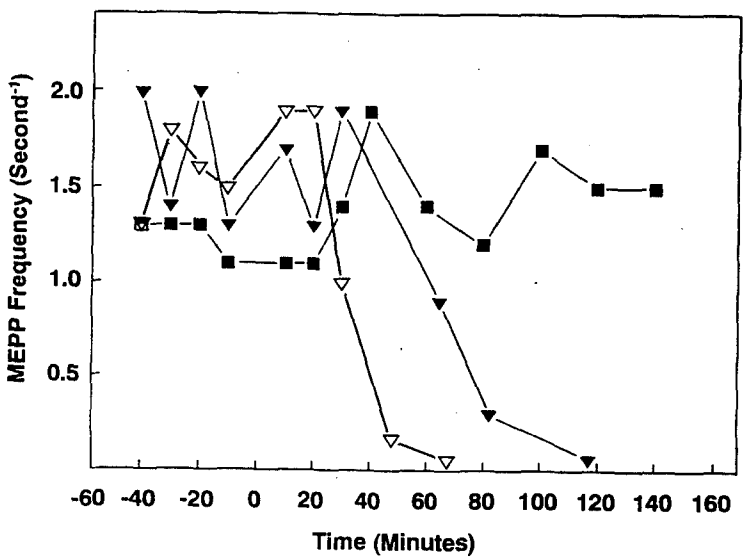


Fig. 5. The effect of pretreatment with chelators on the frequency of spontaneous MEPPs in tissues exposed to botulinum neurotoxin type A. Endplate activity was recorded for a base-line period of 30 min to 60 min in control tissues (▽) and in tissues pretreated with DTPA (▼; 40 μM; 120 min) or TPEN (■; 20 μM; 120 min). MEPPs were recorded for an additional period of time after tissues were exposed to toxin (1×10^{-10} M). A quantitative analysis of the data is presented in the text.

VIII. STUDIES ON VACUOLAR ATP

A. RESULTS

Nerve, muscle and neuromuscular transmission. Two types of experiments were done to gauge the effects of bafilomycin on neuromuscular transmission. In the first, various concentrations of bafilomycin were added to tissues at 35° C, and muscle twitch responses to nerve stimulation (0.1 Hz) were monitored for 250 min. The results indicated that the drug produced concentration-dependent blockade (Fig. 1A). In the second experiment, a single concentration of the drug that was equivalent to the IC50 (5×10^{-6} M) was added to tissues for varying times (0 to 60 min). The tissues were then washed free of drug, after which responses were monitored for at least 250 min. The results demonstrated that bafilomycin required only a relatively short incubation period to produce its effects (~ 30 min; Fig. 1B), even though the effects themselves were not fully manifested for 250 min.

The nature of the blockade produced by bafilomycin was the same, regardless of whether the drug was present constantly or present only for an initial incubation period. In both cases, transmission remained normal immediately after addition of drug. There was a lagtime before onset of blockade, and the duration of the lagtime was inversely proportional to the concentration of drug. Following the lagtime, there was slow and progressive development of blockade. For example, after addition of bafilomycin at a concentration of 5×10^{-6} M, transmission remained normal for approximately 60 to 90 min. Responses then began to diminish, and by approximately 250 min responses were reduced by 50 percent.

In related experiments, tissues were exposed constantly to bafilomycin at a concentration of 1×10^{-5} M. Phrenic nerves were stimulated and twitch responses were monitored until transmission was at least 60% blocked (ca. 180 to 220 min). Individual nerve and muscle fibers were then impaled to determine membrane potential and tissue excitability. Interestingly, phrenic nerve-hemidiaphragm preparations exposed to concentrations of bafilomycin that substantially blocked neuromuscular transmission had normal resting membrane potentials and propagated normal action

potentials.

The effect of bafilomycin on toxin-induced neuromuscular blockade. Bafilomycin (1 x 10^{-6} M) was added to tissues for 30 min, after which botulinum neurotoxin type A (1 x 10^{-11} M) or β-bungarotoxin (1 x 10^{-8} M) was added. Nerves were stimulated at 0.1 Hz, and onset of paralysis (90% blockade) was monitored.

The data revealed a striking difference between the prototype clostridial neurotoxin and the prototype phospholipase A2 neurotoxin. Control tissues (n = 5) exposed only to botulinum neurotoxin paralyzed in 127 $\pm$ 13 min; experimental tissues (n = 5) exposed to bafilomycin and botulinum neurotoxin did not paralyze within 250 min. Control tissues (n = 3) exposed only to β-bungarotoxin paralyzed in 121 $\pm$ 14 min; experimental tissues exposed to bafilomycin and β-bungarotoxin paralyzed in 131 $\pm$ 12 min.

To compensate for the substantial protection that bafilomycin provided against a clostridial neurotoxin, and to ensure that paralysis times fell within 250 min, the experimental approach was altered. Higher concentrations of toxin were used to produce more rapid onset of poisoning, and paralysis was defined as 50% rather than 90% blockade of stimulus-evoked muscle twitch. This approach was used with a single concentration of bafilomycin (1.5 x 10^{-6} M) and with 3 or 4 concentrations each of botulinum neurotoxin type A, botulinum neurotoxin type B and tetanus toxin. The results, which are shown in Figure 2, indicate that bafilomycin diminished the apparent potency of botulinum neurotoxin type A by two orders of magnitude or greater. The effect of bafilomycin on botulinum neurotoxin type B and tetanus toxin appeared to be somewhat less, but the apparent reduction in potency was still in the range of one-to-two orders of magnitude.

Similar experiments were done with the remaining clostridial neurotoxins, except that a single concentration was used with each toxin (serotypes of botulinum toxin, 1 x 10^{-9} M; tetanus toxin, 1 x 10^{-6} M). The results demonstrated that bafilomycin was a universal antagonist of clostridial neurotoxins (Fig. 3). By contrast, bafilomycin did not protect tissues against notexin , β-

bungarotoxin, taipoxin or textilotoxin (Fig. 3).

Timecourse of the interaction between bafilomycin and botulinum neurotoxin type A. Experiments were done to clarify the nature of the interaction between bafilomycin and clostridial neurotoxins. Botulinum neurotoxin type A was selected as a prototype clostridial toxin.

Botulinum toxin (1×10^{-11} M) was added to phrenic nerve-hemidiaphragm preparations, and bafilomycin (1.5×10^{-6} M) was added at various times before or after the toxin. Phrenic nerves were stimulated at a rate of 0.1 Hz, and paralysis was measured either as a 50% or as a 90% reduction in the evoked twitch response. The results (Fig. 4) showed that bafilomycin was fully active when added before or added simultaneously with toxin. The drug was also active when added after the toxin, but the magnitude of effect diminished with time. If bafilomycin was added more than 30 to 40 min after the toxin, it provided little or no protection against the toxin.

These results appear to indicate that bafilomycin did not act by antagonizing toxin binding. This possibility was tested directly in the next set of experiments. Tissues ($n = 5$ per group) were incubated with botulinum neurotoxin type A (1×10^{-11} M) at 4°C and in the absence of nerve stimulation. At the end of incubation (60 min), tissues were washed to remove unbound toxin. Temperature was raised to 35° C, and bafilomycin (1.5×10^{-6} M) was added. After an additional 30 min, nerves were stimulated (0.1 Hz) and paralysis was monitored (50% blockade). Tissues that were treated only with toxin became paralyzed in 97 ± 12 min. Tissues that were exposed to toxin and subsequently treated with bafilomycin became paralyzed in 201 ± 12 min.

In the final set of experiments, the binding of iodinated botulinum neurotoxin type A to brain membrane preparations was studied in the presence and absence of bafilomycin (2×10^{-6} M). Two positive controls were included for comparison: *i.*) unlabeled botulinum neurotoxin type A, and *ii.*) *Triticum vulgaris* lectin, an agent that has previously been shown to antagonize the binding and biological activity of all clostridial neurotoxins (Bakry et al., 1991b). As expected, both homologous unlabeled toxin and lectin antagonized the binding of botulinum neurotoxin type A (Fig. 5). By contrast, a concentration of bafilomycin that provides substantial protection against

the neuromuscular blocking properties of clostridial toxins (e.g., Fig. 3) did not produce detectable antagonism of toxin binding.

Bafilomycin and spontaneous miniature endplate potentials. Spontaneous potentials were monitored in control tissues and in experimental tissues treated with bafilomycin (2×10^{-6} M). During a baseline period of 40 min, bafilomycin neither increased nor decreased the rate of spontaneous potentials.

Botulinum neurotoxin type A (1×10^{-9} M) was added to control tissues and bafilomycin-pretreated tissues, and responses were monitored for an additional 240 min. When tissues were treated only with botulinum neurotoxin type A, the rate of spontaneous potentials fell from basal levels of approximately 3 per second to final levels of approximately 1 per min (Fig. 6). This decrease occurred over a period of approximately 60 min. When tissues were pretreated with bafilomycin and then treated with botulinum neurotoxin, the rate of spontaneous miniature endplate potentials showed little change over a period of 240 min (Fig. 6).

B. COMMENT

There are compelling data to show that clostridial toxins act in the cell interior to block transmitter release, and there are strongly suggestive data to indicate that internalization is via receptor-mediated endocytosis and pH-induced translocation (see Introduction for references). The data on site and mechanism of action of phospholipase A2 neurotoxins are less compelling. However, a hypothesis has been advanced that envisions these toxins inserting into and then acting upon the plasma membrane (Simpson et al., 1993). This hypothesis does not involve receptor-mediated endocytosis or pH-induced translocation.

The proposed mechanisms of clostridial neurotoxin action and phospholipase A2 neurotoxin action give rise to two contrasting predictions. Inhibition of vacuolar ATPase, which will produce inhibition of the proton pump in endosome membranes, should delay or block translocation of clostridial neurotoxins. This would delay or block the neuromuscular blocking properties of

botulinum toxin and tetanus toxin. However, inhibition of vacuolar ATPase should not block translocation of phospholipase A2 neurotoxins and should not antagonize their neuromuscular blocking effects.

These predictions have been tested by using bafilomycin A1, a microbial product that acts selectively to inhibit vacuolar ATPase, and the results indicate that bafilomycin is an antagonist of clostridial neurotoxins. It delays the expression of toxicity due to all seven serotypes of botulinum neurotoxin as well as tetanus toxin. However, bafilomycin has no protective effect against a representative group of four phospholipase A2 neurotoxins.

Bafilomycin action. Dose-response experiments with bafilomycin revealed that the drug produced blockade of neuromuscular transmission, and this drug-induced effect had two interesting properties. First, there was a latent period before the effect became obvious; and second, nerve and muscle transmission remained normal even when neuromuscular transmission was blocked. Additional work is needed to clarify the mechanism of bafilomycin-induced neuromuscular blockade, but there is one possible explanation that seems especially worthy of consideration. Bafilomycin has been shown to act on endosomal, lysosomal and other forms of vacuolar ATPase (Bowman et al., 1988; Yoshimori et al., 1991; Arai et al., 1993; Dröse et al., 1993). This suggests that the drug would also act on granular and vesicular ATPase (see Hanada et al., 1993). If this were true, one would predict that inhibition of the proton pump in vesicular membranes should lead to failure of transport of acetylcholine, and thus to failure of transmission (Amara and Kuhar, 1993). This concept is appealing, because it is in keeping with the characteristics of bafilomycin-induced blockade. There was a latent period before onset of drug effect, and this would be expected. Vesicles that are filled with transmitter before addition of drug should continue to discharge their contents. Blockade should not occur until some fraction of the vesicles had progressed through the cycle of discharging their contents and then failing to become re-filled. Another characteristic of the drug was that it blocked neuromuscular transmission without blocking nerve or muscle transmission. This too was an expected outcome. A drug that acts selectively on vacuoles should impair vesicle membrane function without

impairing plasma membrane function.

One experimental observation appears to support the concept of a drug effect on storage and release of acetylcholine. When the actions of bafilomycin were studied on stimulated preparations, there was significant loss of transmission within 250 min (e.g., Fig. 1). By contrast, when the actions of equivalent concentrations of bafilomycin were studied on unstimulated preparations, there was little or no evidence of blockade (e.g., Fig. 6). Usage-dependent onset of blockade could be explained on the basis that stimulation depletes nerve endings of filled vesicles and hastens the appearance of empty vesicles. This idea is currently being investigated in greater detail.

The fact that bafilomycin exerted significant effects on neuromuscular transmission made it necessary to design a protocol that would allow for observation of any putative antagonism of clostridial neurotoxins or phospholipase A2 neurotoxins. This was achieved by using submaximal concentrations of the drug, by employing high concentrations of toxin, and by defining blockade as a 50% reduction in the evoked muscle response or a 50% reduction in the rate of spontaneous miniature endplate potentials. Under these conditions, bafilomycin was a very effective antagonist of all serotypes of botulinum neurotoxin and tetanus toxin. It shifted the dose-response curves to the toxins by orders of magnitude. Bafilomycin did not have a comparable effect on the potencies of notexin, β-bungarotoxin, taipoxin or textilotoxin.

Site of protection. Clostridial neurotoxins proceed through a series of steps to produce their paralytic effects, including a binding step, an internalization step, and an intracellular poisoning step. Therefore, experiments were designed to identify the step(s) antagonized by bafilomycin.

The data strongly suggest that the drug does not interfere with binding. Two pieces of evidence support this conclusion. First, bafilomycin provided substantial protection against the neuromuscular blocking properties of the toxin, even when the drug was added to tissues after sufficient time for toxin binding to be complete (Simpson, 1980). Second, bafilomycin did not antagonize the binding of labeled toxin to nerve membrane preparations. The data also suggest

that bafilomycin does not alter the intracellular actions of the toxin. Thus, bafilomycin acted as an antagonist when added to tissues simultaneously with toxin, but it was not an antagonist when added approximately 30 min after the toxin. This is an interesting finding, because it means that bafilomycin lost its ability to behave as an antagonist well before the toxin expressed its full intracellular effects.

The failure of the drug to act as an antagonist when added after the toxin cannot be due to a lack of time to reach its site of action. This conclusion is supported by a comparison of the data in Figure 2 and Figure 4. As Figure 2 illustrates, high concentrations of toxin (10^{-8} to 10^{-9} M) produced paralysis within approximately 20 min. When bafilomycin was added 30 min prior to toxin, there was significant protection. Therefore, the maximum amount of time needed for the drug to reach its site of action and express its effects was 50 min, and probably less. As Figure 4 illustrates, lower concentrations of toxin (10^{-11} M) produced paralysis in 100 min or longer. However, when bafilomycin was added 30 min after the toxin, the drug did not provide protection even though an additional 70 to 80 min (50% blockade) or 90 to 110 min (90% blockade) was needed for toxin-induced paralysis. Thus, the failure of the drug to provide protection when added shortly after the toxin cannot be explained on the basis that bafilomycin failed to reach its site of action. More plausibly, the failure was due to the fact that the toxin had progressed beyond the site of bafilomycin action.

Both the timecourse of the interaction between bafilomycin and toxin, as well as the known action of bafilomycin on vacuolar ATPase, suggest that the drug was antagonizing the intermediate step of internalization. A protective effect that was limited to the internalization step would be in keeping with acid-dependent translocation of toxin to cytosol. By inhibiting endosomal ATPase, bafilomycin would block intraluminal acidification and therefore block translocation. An action that was limited to the internalization step would also explain the time-dependent effects of bafilomycin. Toxin that had sufficient time to escape endosomes would be beyond the site at which bafilomycin could provide protection.

Mechanism of tetanus toxin action. The experiments with bafilomycin carried an added

benefit that pertains solely to tetanus toxin. The data may provide insights into the mechanism that underlies tetanus toxin-induced neuromuscular blockade.

During the natural course of poisoning at low concentrations, tetanus toxin enters peripheral nerves and is conveyed by transport vesicles to the central nervous system where it blocks release of inhibitory neurotransmitters (Wellhöner, 1982; Habermann and Dreyer, 1986). However, when tested at high concentrations, tetanus toxin enters peripheral nerves and acts locally to block transmitter release (Habermann et al., 1980). There are two mechanisms that could account for the ability of tetanus toxin to act locally to block neuromuscular transmission. One possibility is that, when tested at high concentrations, tetanus toxin overloads its normal mechanism for endocytosis and transport to the central nervous system. An excess of toxin might cause transport vesicles to lyse, thus producing local release of toxin. The other possibility is that, when tested at high concentrations, some portion of the excess toxin enters endosomes that mediate local delivery of substances. When these endosomes undergo acidification, the toxin is released locally into nerve endings.

The finding that bafilomycin antagonizes the neuromuscular blocking properties of tetanus toxin is a strong indication that the toxin is not escaping from transport vesicles that normally convey it to the central nervous system. If these vesicles possessed a mechanism for intraluminal acidification, tetanus toxin would be a potent neuromuscular blocking agent. A more likely explanation is that high concentrations of tetanus toxin bind to receptors other than those that mediate natural poisoning. These receptors are linked to endosomes that are acidified locally. Thus, when bafilomycin inhibits acidification of endosomes it also inhibits the local actions of high concentrations of tetanus toxin.

Pharmacological antagonism of clostridial neurotoxins. There is a rapidly growing literature on drugs that antagonize botulinum neurotoxin and tetanus toxin (Simpson, 1993). This literature indicates that pharmacological antagonists should be divided into two classes: differential (or selective) and universal. Differential antagonists are those that delay the paralytic effects of some clostridial neurotoxins but not others. Universal antagonists are drugs that

significantly alter the biological activity of all serotypes of botulinum neurotoxin as well as tetanus toxin.

There are three groups of universal antagonists that have been identified to date. Lectins with affinity for sialic acid act at the level of the plasma membrane to antagonize toxin binding to receptors (Bakry et al., 1991b). Ammonium chloride, methylamine hydrochloride and chloroquine act at the step of productive internalization to antagonize toxin delivery to the cytosol (Simpson, 1982; Simpson, 1983). Chelators of zinc act inside the cell to antagonize the putative metalloendoprotease activity of toxins (Schiavo et al., 1992; Simpson et al., in press).

The data in the present study demonstrate that bafilomycin A1 is a universal antagonist of clostridial neurotoxins, although it is not an antagonist of phospholipase A2 neurotoxins. The drug shares with ammonium chloride, methylamine hydrochloride and chloroquine the ability to antagonize internalization, but it has a decided advantage. There remains uncertainty about the precise mechanism of action of the drugs that were formerly shown to prevent internalization, but bafilomycin has a well described mechanism of action (see above). This should facilitate the effort to identify new and potentially better toxin antagonists. And this in turn should enhance the effort to find a clinically useful drug or mixture of drugs that will block clostridial neurotoxin action on human neuromuscular junctions.

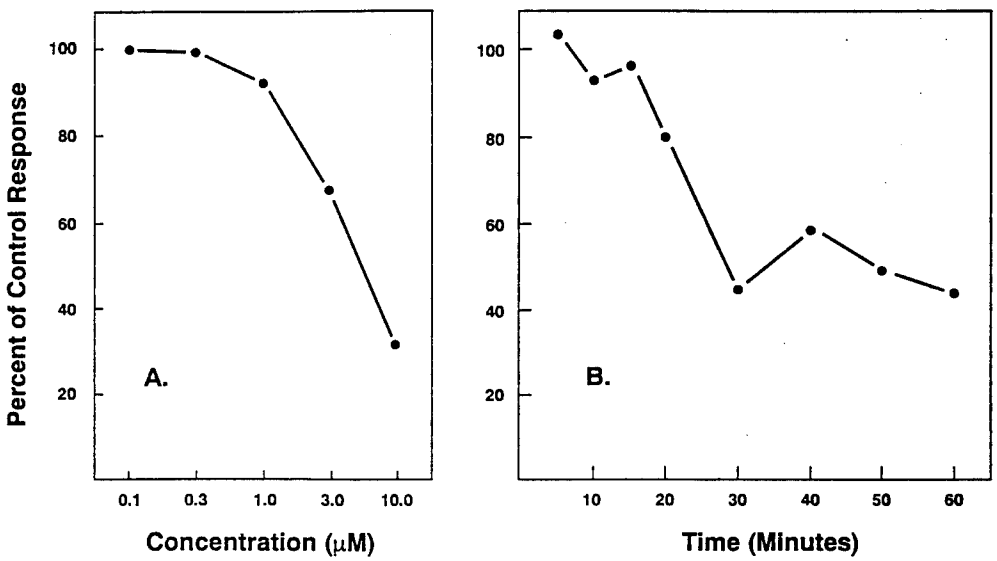


Fig. 1. Two experimental paradigms were used to determine the effects of bafilomycin: 1) constant exposure to various concentrations of the drug (A) or 2) varying exposures to a single concentration of drug (IC_{50}; 5 μM; B). In both cases, phrenic nerves were stimulated and muscle twitch responses were monitored for at least 250 min after addition of drug. The results indicate that bafilomycin produced concentration-dependent blockade of neuromuscular transmission. The results also show that the drug-induced effect was obtained even when exposure to the drug was of limited duration (~30 min). Data points for both portions of the figure represent the mean of four or more observations. The S.E.M. for each data point is equal to or less than 13% of the respective mean.

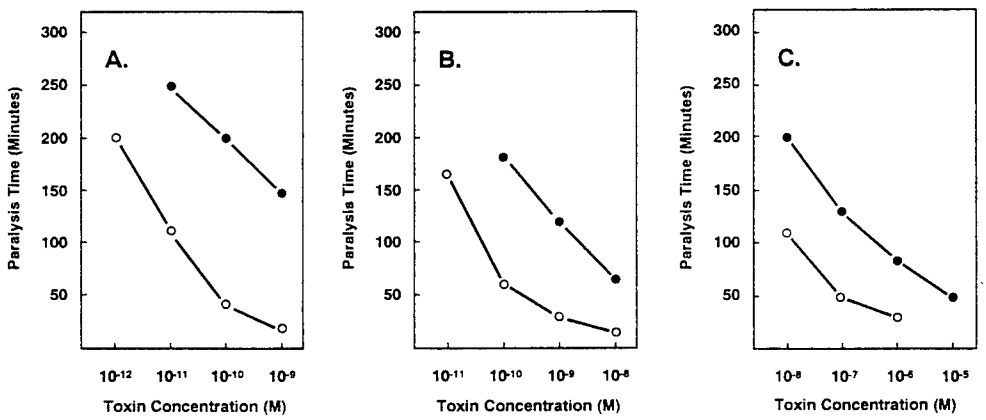


Fig. 2. Tissues were pretreated with 1.5 μM bafilomycin at 35°C for 30 min, then treated with various concentrations of botulinum neurotoxin type A (A), botulinum neurotoxin type B (B) or tetanus toxin (C). The paralysis times for these tissues were compared with those of tissues that were not pretreated with the drug. The results showed that tissues pretreated with bafilomycin (●) required substantially more time to become paralyzed (50% blockade) than tissues not pretreated with the drug (○). Data points for each portion of the figure represent the mean of five or more observations, and the S.E.M. for each data point is equal to or less than 15% of the respective mean. The average paralysis time for tissues at each toxin concentration in the presence of drug was significantly longer ($P < .01$) than the average paralysis time for tissues in the absence of drug.

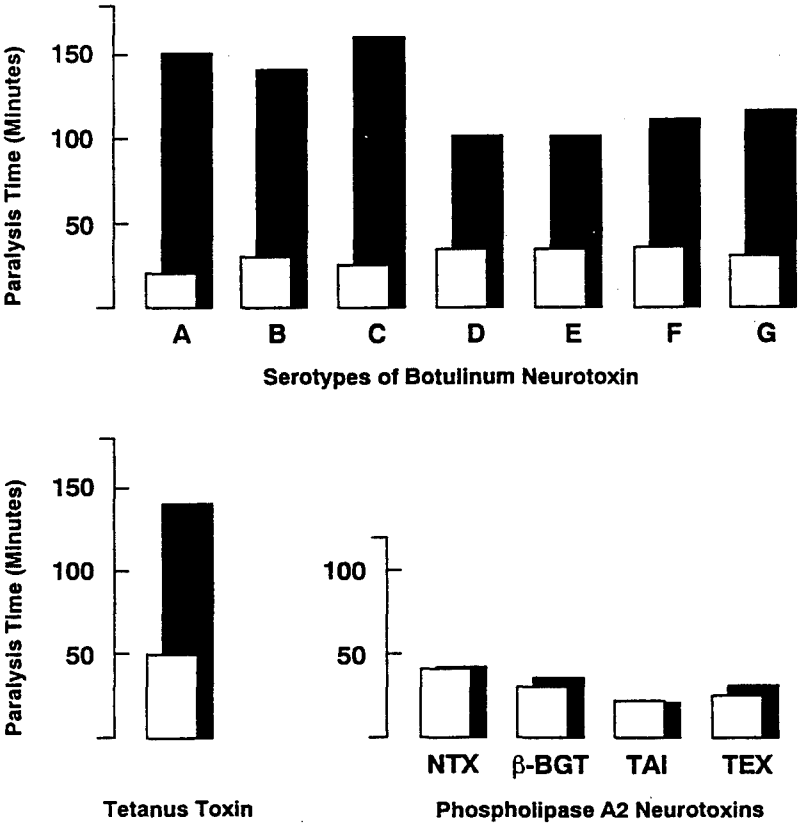


Fig. 3. Tissues were pretreated with 1.5 μM bafilomycin at 35°C for 30 min, then treated with a single concentration of botulinum neurotoxin types A to G, tetanus toxin or phospholipase A2 neurotoxins. The paralysis times for these tissues (■) were compared with those of tissues not pretreated with drug (□). The results showed that experimental outcome depended on the toxins under study. Bafilomycin pretreatment provided substantial protection against all seven serotypes of botulinum neurotoxin (1×10^{-9} M) and tetanus toxin (1×10^{-6} M), but it provided no protection against phospholipase A2 neurotoxins (notexin, NTX; β-bungarotoxin, β-BGT; taipoxin, TAI; and textilotoxin, TEX; each at 1×10^{-6} M). Each bar represents the mean of four or more observations, and the S.E.M. for each bar is equal to or less than 9% of the respective mean.

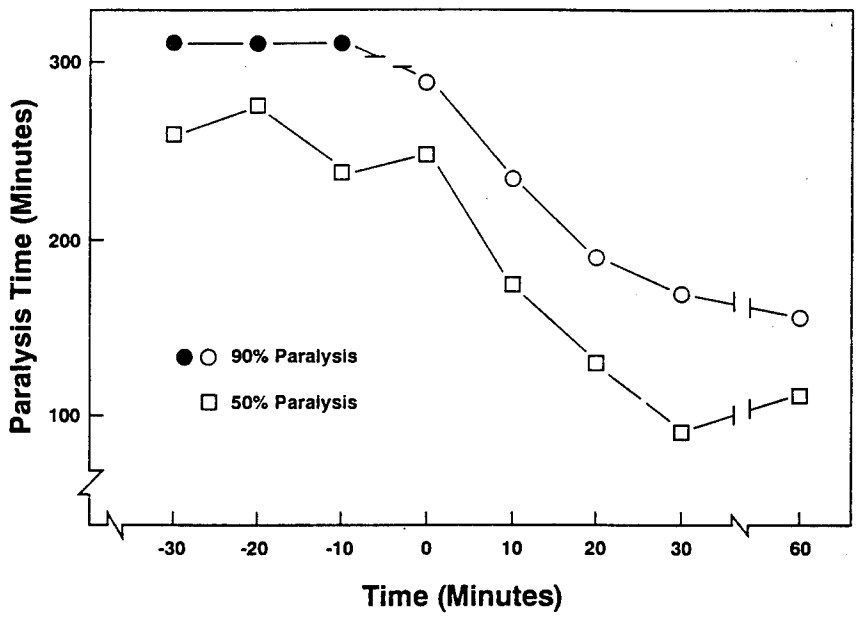


Fig. 4. Bafilomycin (1.5×10^{-6} M) was added to tissues at various times before or after botulinum neurotoxin type A (1×10^{-11} M), and paralysis times were monitored (50% blockade, □; 90% blockade, ○). The results showed that the drug provided protection when added before the toxin or with the toxin, but the drug had a diminishing effect when added at various times after the toxin. Each data point represents the mean of three or more observations. The S.E.M. for data points reflecting paralysis times of 300 min or less were equal to or less than 17% of the respective mean. No attempt was made to quantify paralysis times of greater than 300 min, and thus no S.E.M. values were determined for these data points (●).

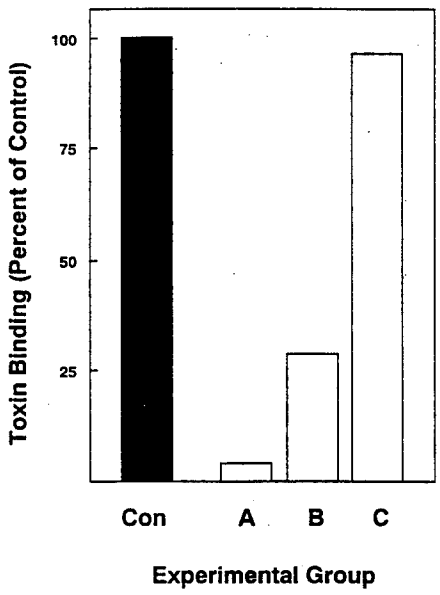


Fig. 5. The binding of iodinated botulinum neurotoxin type A (5×10^{-10} M) was studied in four groups: 1) control tissues (Con); 2) tissues pretreated with a molar excess of unlabeled toxin (1×10^{-7} M; 90 min; A); 3) tissues pretreated with *Triticum vulgaris* lectin (3×10^{-5} M; 30 min; B); and 4) tissues pretreated with bafilomycin (2×10^{-6} M; 30 min; C). The results demonstrated that bafilomycin did not antagonize toxin binding to nerve membrane preparations. The data reflect the means of two experiments done in quadruplicate.

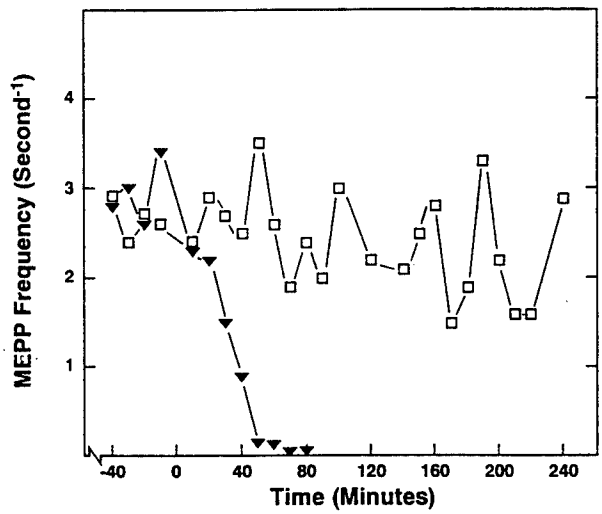


Fig. 6. Spontaneous miniature endplate potentials (MEPP) were monitored in control tissues (▼) and in tissues pretreated with bafilomcyin (1.5×10^{-6} M; □). The base-line frequency of spontaneous potentials was monitored for 40 min, after which botulinum neurotoxin type A was added (1×10^{-9} M). The base-line frequency in control tissues was 2.7 ± 0.14 per sec, and after addition of toxin the frequency fell to less than 0.1 per sec. The base-line frequency in bafilomycin tissues was 2.6 ± 0.19 per sec. The frequency varied after addition of toxin, but over a period of 240 min there was no evidence for onset of paralysis. Each data point represents the average result from three muscles, and five or more endplates were sampled (1–2 min) per muscle.

IX. STUDIES ON TRANSGLUTAMINASE

A. RESULTS

Sequence homology and substrate activity. There are two domains in tetanus toxin that reportedly have sequence homology with transglutaminase substrates and thus are presumably sites of transglutaminase-induced cross-linking (Facchiano and Luini, 1992). It is interesting that these two domains do not have significant homology with one another (Fig. 1).

One of the domains that reportedly has substrate homology is found in the light chain and the other is in the heavy chain of the toxin. It should be noted that only the light chain is essential for the intracellular actions that culminate in blockade of exocytosis (Bittner et al., 1989; Ahnert-Hilger et al., 1989).

The primary sequence of the light chain of tetanus toxin was aligned with the primary sequences of the light chains of botulinum neurotoxin types A to E. This was done to determine whether there was homology in the putative transglutaminase substrate domain (Fig. 1). Of the five botulinum neurotoxins, only type B possessed both sequence homology with tetanus toxin and an essential glutamine. Serotype A possessed weak homology and a misaligned glutamine. The other three serotypes possessed variable homology and no reactive glutamine.

The light chains of tetanus toxin and botulinum neurotoxin types A to E were searched to determine whether there were other domains in which the group shared sequence homology and a reactive glutamine. The results, which are shown in Figure 1, indicate that there was a region in the carboxyterminus of the molecules that possessed an aligned glutamine. However, recent evidence indicates that this portion of the toxin molecule is not essential for blockade of exocytosis (Kurazono et al., 1992).

The alignment data indicate that only tetanus toxin and botulinum neurotoxin type B have domains that could account for substrate activity. Therefore, these two toxins were assayed as substrates for transglutaminase at concentrations that are relevant to blockade of transmitter release

at the neuromuscular junction and in NG-108 cells (10^{-12} M to 10^{-9} M; see below). The results of these experiments were negative. Even when tested at a high concentration (1 x 10^{-9} M), the toxins possessed little if any ability to serve as substrates for transglutaminase-induced cross-linking. This indicates that: *a.)* tetanus toxin and botulinum neurotoxin type B are not important substrates for transglutaminase, and *b.)* to the extent that the toxins are substrates, this is not an important part of the process of blocking transmitter release.

Stimulation of transglutaminase activity. Experiments were done on intact cells and on isolated enzyme preparations to determine whether tetanus toxin or botulinum neurotoxin type B would stimulate transglutaminase at meaningful concentrations. In the initial experiment various concentrations of tetanus toxin were incubated with NG-108 cells for 180 min, after which the extent of toxin-induced blockade of acetylcholine release was measured. The results (Fig. 2) indicated that concentrations in the range of 10^{-12} to 10^{-10} M produced partial to complete blockade of transmitter release.

In the next experiment, NG-108 cells were incubated for 180 min with 1 x 10^{-9} M tetanus toxin. Cells were then ruptured by sonication and exposed to varying concentrations of exogenous calcium (35°C; 180 min). Transglutaminase activity was assayed by quantifying the amount of cross-linked protein in the stacking gel, as described by Barsigian et al. (1988). The results indicated that, even at a concentration that totally blocks exocytosis, tetanus toxin did not alter the pattern or amount of cross-linked protein (Fig. 3). It did not induce the appearance of cross-linked protein at low calcium concentrations (e.g., 100 μM calcium) nor did it increase the amount at high calcium concentrations (e.g., 10 mM).

In the final experiment, tetanus toxin and botulinum neurotoxin type B were examined for their ability to stimulate transglutaminase-mediated incorporation of tritiated putrescine into dimethylcasein. Toxins and transglutaminase were tested at various ratios (0.1 to 1.0; 1.0 to 1.0; 1.0 to 0.1), at various concentrations (maximum, 1 x 10^{-9} M), and for various lengths of time (30, 60 and 120 min). There was no paradigm in which the toxins produced a statistically significant increase in the amount of tritiated putrescine incorporated into dimethylcasein.

Clostridial toxins, transglutaminase and neuromuscular transmission. Transglutaminase is a calcium dependent enzyme. As gauged by incorporation of putrescine into dimethylcasein, the EC50 for calcium was in the range of 10 to 100 μM (results not illustrated). Magnesium and strontium possessed no more than 10% of the activity of calcium in supporting transglutaminase activity.

The level of cytosolic calcium in quiescent nerves is in the range of 100 to 300 nM (Blaustein, 1988), which is approximately two orders of magnitude below the EC50 for calcium supported transglutaminase activity. Therefore, the actions of tetanus toxin and botulinum neurotoxin type B were studied on quiescent nerves. To further ensure that cytosolic calcium levels did not rise, the experiments were repeated with quiescent nerves suspended in medium in which calcium was replaced with equimolar concentrations of magnesium or strontium.

Interestingly, both toxins blocked transmission when added to tissues under conditions that would not be expected to support transglutaminase activity (n=3 or more per group). When tetanus toxin (1×10^{-9} M) was added to unstimulated phrenic nerve-hemidiaphragm preparations in physiological medium, the average paralysis time was 172 ± 9 min. Similarly, when botulinum neurotoxin type B (1×10^{-12} M) was added to preparations in physiological medium, the average paralysis time was 191 ± 12 min. The results for both toxins were not significantly different when calcium was replaced by magnesium or strontium.

As a further test of the involvement of transglutaminase in clostridial toxin action, experiments were done in the presence of glycine methylester and monodansylcadaverine. These agents can serve as false substrates for the enzyme, and thus they can be used to inhibit the cross-linking effects of endogenous substrates by transglutaminase (Pastuszko et al., 1986; Bungay et al., 1984; Senner et al., 1985).

Glycine methylester and monodansylcadaverine were assayed for their ability to inhibit transglutaminase-mediated incorporation of tritiated putrescine into dimethylcasein. The respective IC50 values were: glycine methylester, 2×10^{-4} M; monodansylcadaverine, 1 x

10^{-5} M. The concentrations of the drugs had to be incremented 2-to-4 fold to obtain comparable effects on intact NG-108 cells.

Glycine methylester and monodansylcadaverine were tested for their abilities to alter stimulus-evoked muscle twitch and spontaneous miniature endplate potentials (group n=3 or more). At concentrations equal to or greater than the IC50 values (glycine methylester, 3 x 10^{-3} M; monodansylcadaverine, 1 x 10^{-5} M), the drugs produced no observable effects on muscle twitch over a period of 120 min. The drugs similarly failed to produce an effect on the frequency of spontaneous miniature endplate potentials. For example, the rate of spontaneous potentials during a baseline period of 30 to 60 min was 135 $\pm$ 22 per min. When tissues were exposed to glycine methylester (3 x 10^{-3} M; 30 min), the frequency was 111 $\pm$ 12 per min. In a similar experiment with monodansylcadaverine (1 x 10^{-5} M) the rate of spontaneous miniature endplate potentials during a baseline period was 99 $\pm$ 14 per min, and the rate during exposure to the drug was 85 $\pm$ 6 per min. These results show that transglutaminase-induced cross-linking of synaptic vesicle proteins does not play an important role in governing the normal process of neuromuscular transmission.

Experiments were done to determine whether transglutaminase inhibitors would alter clostridial neurotoxin-induced blockade of exocytosis. Both mechanical responses and electrophysiological responses were monitored. In studies on stimulus-evoked muscle twitch, neither monodansylcadaverine nor glycine methylester delayed the onset of toxin-induced neuromuscular blockade (Table 1). In studies on electrophysiological responses, the drugs similarly failed to protect tissues against toxin-induced effects (Table 2). These results are difficult to reconcile with the hypothesis that transglutaminase mediates the blocking effects of tetanus toxin.

Sequence homology and proteolytic activity. Tetanus toxin has a highly selective proteolytic action; it cleaves the $Q_{76}F_{77}$ bond in synaptobrevin 2 (Schiavo et al., 1992). The specificity of this reaction is evident in the facts that: *a.)* the toxin does not cleave the QK or QA bonds in synaptobrevin 1 or synaptobrevin 2, and *b.)* it does not cleave the VF bond in synaptobrevin 1.

Numerous forms of transglutaminase have been sequenced in several laboratories, and many of these molecules possess a single QF doublet. Therefore, an effort was made to deduce the importance of the doublet by attempting to produce proteolytic cleavage with tetanus toxin. Transglutaminase and toxin were incubated under conditions similar to those described by Facchiano and Luini (1992), after which they were submitted to polyacrylamide gel electrophoresis. The QF doublet typically exists one-quarter to one-third of the distance from the N-terminus to the C-terminus, and thus proteolysis at this site should be easily detected. As a positive control, tetanus toxin was incubated with synaptobrevin to demonstrate cleavage of this molecule.

The results, which are shown in Fig. 4, confirm the ability of tetanus toxin to cleave synaptobrevin. However, the toxin produced no obvious cleavage of transglutaminase, even when tested at 10^{-7} M.

B. COMMENT

Tetanus toxin is a zinc-binding protein that possesses the properties of a metalloendoprotease. The toxin cleaves a specific peptide bond in synaptobrevin 2, a vesicle associated protein thought to play a role in exocytosis. This action almost certainly contributes to toxin-induced blockade of exocytosis (Schiavo et al., 1992; Link et al., 1992).

An additional action for tetanus toxin has been proposed by Facchiano, Luini and their colleagues, who reported that tetanus toxin stimulates the cross-linking enzyme transglutaminase (Facchiano and Luini, 1992). Stimulation of the enzyme may be due to proteolytic cleavage that converts an inactive precursor to an active product (Facchiano et al., 1993). Therefore, a series of experiments were done to evaluate the possibility that tetanus toxin - or the structurally and functionally similar botulinum neurotoxin type B - exert their effects via transglutaminase.

Sequence analysis data and enzyme~substrate experiments have been interpreted to mean that tetanus toxin is a substrate for transglutaminase (Facchiano and Luini, 1992). However, closer

analysis of the sequence data reveals that evidence for substrate homology is not compelling (Fig. 1). In addition, enzyme-substrate experiments at meaningful concentrations of toxin demonstrated that the latter is not an important substrate.

Tetanus toxin reportedly stimulates transglutaminase to cross-link endogenous proteins, and this stimulation may be due to proteolytic activation of the cross-linking enzyme (Facchiano et al., 1993). It is noteworthy that toxin-induced cross-linking was not shown on intact cells, and toxin-induced cleavage of transglutaminase was not reported (Facchiano et al., 1993).

Experiments in the present study show that tetanus toxin does not produce obvious stimulation of the enzyme nor does it produce obvious proteolysis of the enzyme in an isolated assay system. Furthermore, the toxin does not produce detectable stimulation of cross-linking in intact cells. It was also found that false substrates did not block toxin action on nerve endings, and ambient calcium levels below those needed to support enzyme activity did not prevent paralysis.

In the aggregate, this evidence weighs against the idea that toxin-induced stimulation of transglutaminase is the principal reason for toxin-induced blockade of neurotransmitter release. Nevertheless, there are two cautionary notes that should be added. First, the data do not rule out the possibility that high concentrations of, or lengthy exposure to, tetanus toxin could alter transglutaminase activity. Second, the data do not clarify the mechanism of toxin action on cells in which synaptobrevin does not play a role in exocytosis.

TABLE 1

Effects of drugs on neuromuscular blockade

Mouse phrenic nerve-hemidiaphragm preparations were excised and incubated in physiological solution at 35° C. Phrenic nerves were stimulated (0.1 Hz) and muscle twitch was recorded. Drugs were added to tissue baths 30 min before addition of tetanus toxin (1 x 10^{-9} M) or botulinum neurotoxin type B (1 x 10^{-11} M). Each data point represents the mean ± SEM of four or more preparations.

Toxin	Drug Pretreatment	Paralysis Time
Tetanus toxin	-	131 ± 12
Tetanus toxin	Glycine methylester (3 x 10^{-4} M)	124 ± 11
Tetanus toxin	Glycine methylester (3 x 10^{-3} M)	129 ± 13
Tetanus toxin	Monodansylcadaverine (1 x 10^{-5} M)	126 ± 8
Botulinum toxin	-	117 ± 7
Botulinum toxin	Glycine methylester (3 x 10^{-4} M)	125 ± 11
Botulinum toxin	Glycine methylester (3 x 10^{-3} M)	120 ± 14
Botulinum toxin	Monodansylcadaverine (1 x 10^{-5} M)	119 ± 13

TABLE 2

Effects of false substrates on neuromuscular transmission

Mouse phrenic nerve-hemidiaphragm preparations were excised from animals and handled as described in Table 1, except that miniature endplate responses rather than muscle twitch responses were recorded.

Toxin[a,b]	Drug Pretreatment[b,c]	Spontaneous Miniature Endplate Potentials (min^{-1})
-	-	94 ± 11
+	-	11 ± 2
-	-	135 ± 22
-	Glycine methylester	111 ± 12
+	Glycine methylester	10 ± 2
-	-	99 ± 14
-	Monodansylcadaverine	85 ± 6
+	Monodansylcadaverine	16 ± 1

[a] Tetanus toxin was used at a concentration of 1×10^{-9} M.

[b] The protocol for experiments involving drug pretreatment and subsequent addition of toxin is given under Methods.

[c] Glycine methylester was used at 3×10^{-3} M, and monodansylcadaverine was used at 1×10^{-5} M.

FIGURE CAPTIONS

Figure 1. Alignment of the primary structures of tetanus toxin and botulinum neurotoxin. The upper part of the figure aligns the two domains of tetanus toxin that reportedly have sequence homology with known transglutaminase substrates (5). It is interesting that the only true homology between the two is the reactive glutamine (Q) that is characteristic of transglutaminase substrates. The middle part of the figure aligns the purported substrate domain of the light chain of tetanus toxin with the corresponding regions of the light chains of botulinum neurotoxin types A to E. As the Figure illustrates, only the light chain of botulinum neurotoxin type B has significant sequence homology with the light chain of tetanus toxin. The lower part of the figure shows the only region of the light chains of the six toxins in which there is true alignment of glutamine residues. This region is in the carboxyterminus of the light chains, and it is a portion of the molecule that is not required for blockade of exocytosis (12). The primary structures for the various toxins were obtained as follows: tetanus toxin (17,18), botulinum neurotoxin type A (19,20), type B (21), type C (22), type D (23), and type E (24,25).

Figure 2. Blockade of acetylcholine release from NG-108 cells. Various concentrations of tetanus toxin were incubated with cells that had been preloaded with [methyl ^{14}C]choline chloride. After 180 min, cells were depolarized and the medium was assayed for radioactive acetylcholine. Complete blockade of exocytosis was obtained with 180 min exposure to 10^{-10} M toxin.

Figure 3. Effect of tetanus toxin on transglutaminase-mediated cross-linking of endogenous proteins in NG-108 cells. Two experimental paradigms were used, as follows: *i.)* cells were homogenized and assayed for enzyme-induced cross-linking of proteins in the absence of toxin (lanes 1 to 4), or *ii.)* tetanus toxin was pre-incubated with cells (1×10^{-9} M;

180 min), after which cells were washed, homogenized, and assayed for enzyme-induced cross-linking (lanes 5 to 8). In all cases, lysates were incubated for 180 min before being submitted to polyacrylamide gel electrophoresis.

Assays were done in the presence of varying concentrations of calcium, as follows: lanes 1 and 5, no calcium; lanes 2 and 6, 100 μM calcium; lanes 3 and 7, 1 mM calcium; and lanes 4 and 8, 10 mM calcium. Note that pretreatment of cells with toxin did not increase the amount of cross-linked protein in the stacking gel, nor did it decrease the amount of free protein in the resolving gel. The location and approximate molecular weights of the heavy and light chains of tetanus toxin are indicated. The location of synapsin I is between the heavy and light chains of the toxin (doublet; ~86KD and 80KD).

Figure 4. Effect of tetanus toxin on putative substrates. Transglutaminase was incubated with, and then separated from, tetanus toxin as described in the text. The isolated enzyme (~3 μg protein) was applied to a 7.5% gel and stained with Comassie (Panel A). Lane 1, 0°C for 1 hr (control); Lane 2, 37°C for 1 hr. ^{35}S-Synaptobrevin was also incubated with tetanus toxin as described in the text (37°C for 30 min). The mixtures were applied to a 15% gel and then submitted to autoradiography (Panel B). Lane 1, ^{35}S-synaptobrevin; Lane 2, ^{35}S-synaptobrevin plus toxin (1 x 10^{-7} M); Lane 3, ^{35}S-synaptobrevin plus toxin (1 x 10^{-8} M); Lane 4, ^{35}S-synaptobrevin plus toxin (1 x 10^{-9} M). Note that the toxin did not cleave either of the bands associated with transglutaminase, but it produced concentration-dependent cleavage of synaptobrevin.

FIGURE 1

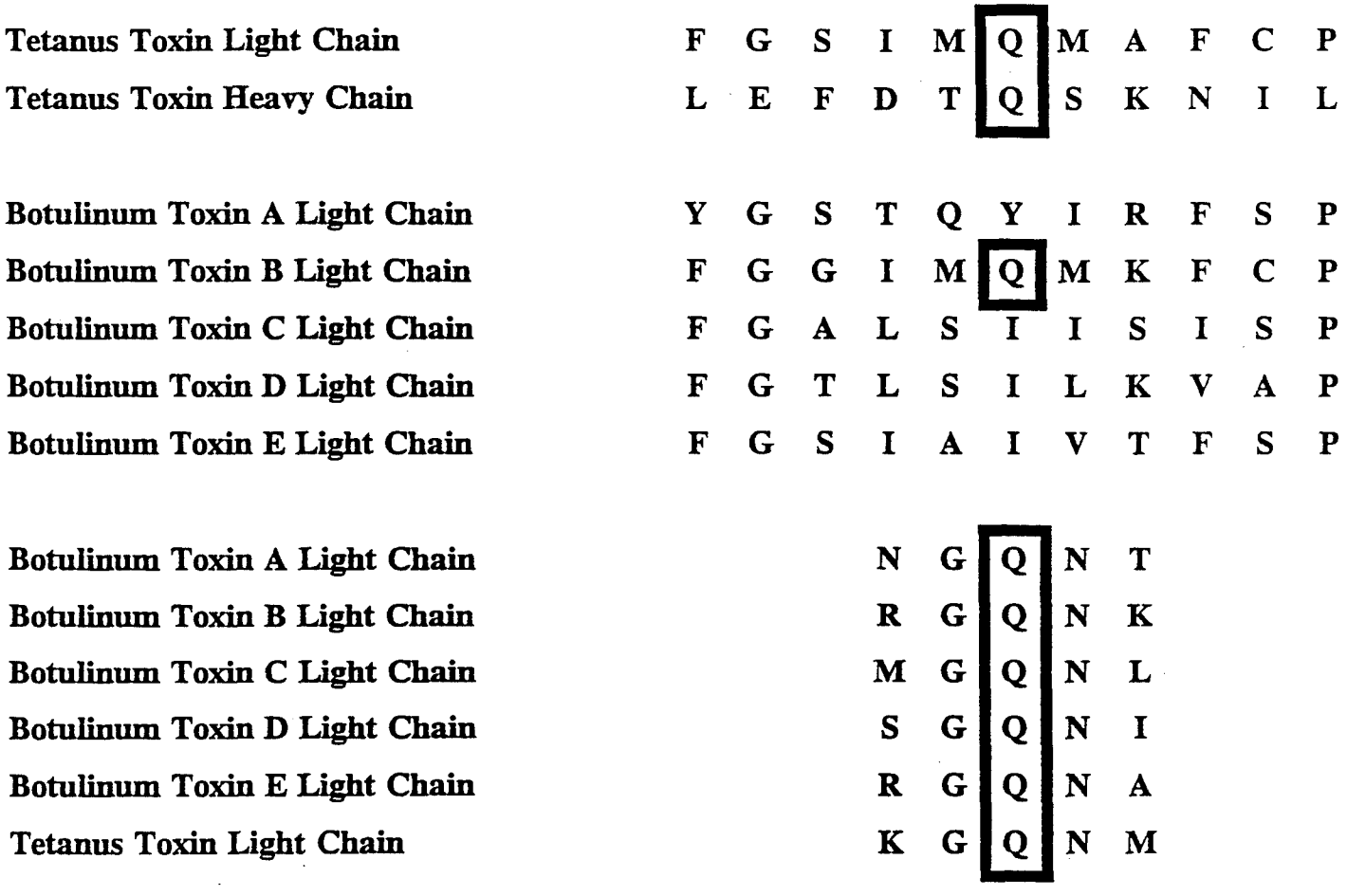

Tetanus Toxin Light Chain F G S I M Q M A F C P
Tetanus Toxin Heavy Chain L E F D T Q S K N I L
Botulinum Toxin A Light Chain Y G S T Q Y I R F S P
Botulinum Toxin B Light Chain F G G I M Q M K F C P
Botulinum Toxin C Light Chain F G A L S I I S I S P
Botulinum Toxin D Light Chain F G T L S I L K V A P
Botulinum Toxin E Light Chain F G S I A I V T F S P
Botulinum Toxin A Light Chain N G Q N T
Botulinum Toxin B Light Chain R G Q N K
Botulinum Toxin C Light Chain M G Q N L
Botulinum Toxin D Light Chain S G Q N I
Botulinum Toxin E Light Chain R G Q N A
Tetanus Toxin Light Chain K G Q N M

FIGURE 2

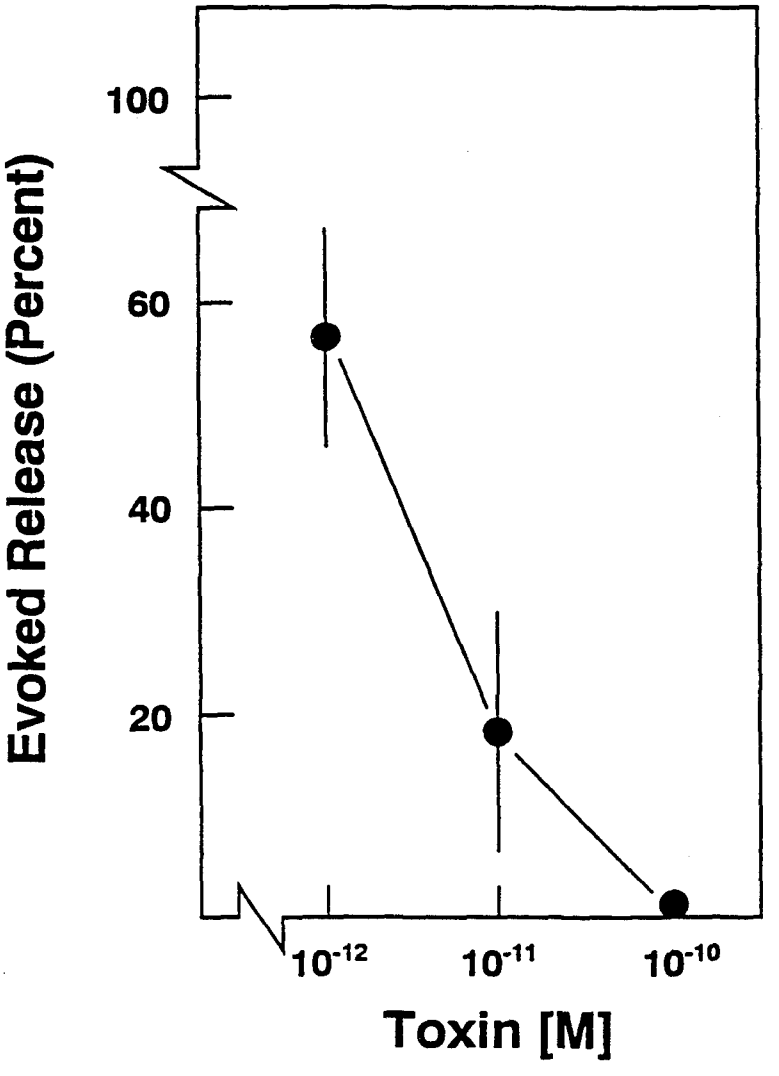


FIGURE 3

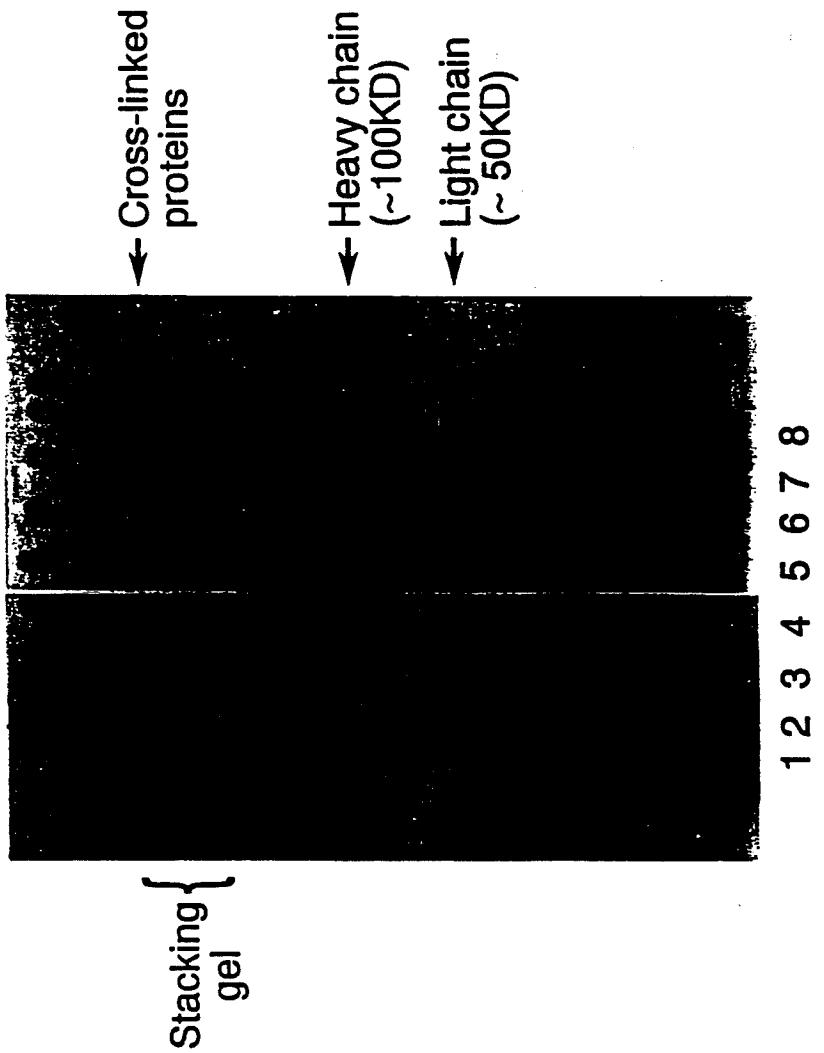
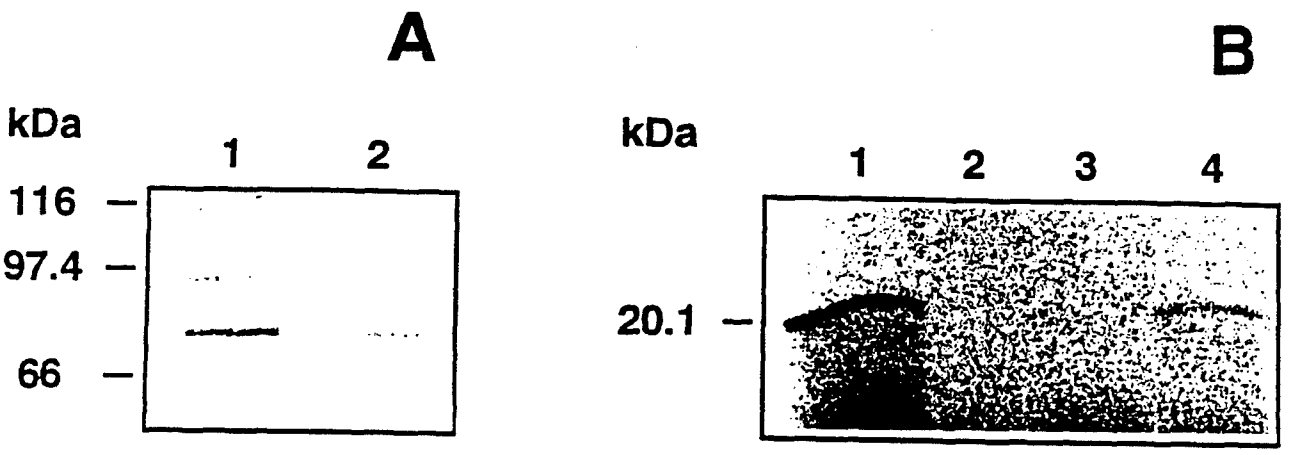

Cross-linked proteins
Heavy chain (~100KD)
Light chain (~ 50KD)
1 2 3 4 5 6 7 8
Stacking gel

FIGURE 4



X. STUDIES ON HUMAN PYRAMIDALIS MUSCLE

A. RESULTS

Initial experiments on the human neuromuscular junction were done with tissues from a variety of surgical sources. These included muscle biopsies from various parts of the body, intercostal muscle obtained during thoracic surgery, and facial muscle obtained during plastic surgery. These tissues were used in an effort to determine the sensitivity of human neuromuscular junctions to the actions of botulinum toxin.

Although these initial experiments appeared to be promising, it is now clear that - at least in part - they were yielding inconsistent and at times misleading results. The major problem was the variable thickness of the muscle preparations. Due to the diffusion barriers posed by thick muscles, a failure of any particular serotype to produce neuromuscular blockade could not be viewed with confidence. Therefore, it was necessary to re-initiate the work, and to do so by focusing on a single muscle type that would be relatively consistent in size and thickness from experiment-to-experiment. This was achieved by utilizing the pyramidalis muscle. This tissue is somewhat more available from surgery than most other individual striate muscles, although certainly less available that all striate muscles combined. The implication is that there was an experimental balance that was struck: there were fewer tissues available than previously when all surgical specimens were used, but the consistency of the data increased dramatically.

Table 1 presents a summary of data describing some of the electrophysiological characteristics of the human pyramidalis muscle. The muscle had a normal resting membrane potential (RMP), and this was well maintained over time and in the presence of mild potassium depolarization. The latter point is important, because of the nature of the experimental protocol. There was not an adequate length of nerve attached to surgically excised pyramidalis muscle to allow for evoking of indirect responses. As an alternative, the rate of spontaneous miniature endplate potentials (MEPP's) was measured. This was facilitated by slightly incrementing the potassium concentration and thereby increasing the rate of spontaneous MEPP's (Table 1). Raw data

illustrating the actual rate of MEPP's in a representative muscle are shown in Figure 1. The Figure shows MEPP's at a potassium level of: *i.)* 4.0 mM (a), *ii.)* 12.5 mM (b), and *iii.)* 25 mM (c). The summed and averaged data for endplates in 19 tissues are shown in Figure 2.

The rate of spontaneous MEPP's was then monitored in a series of control tissues and in experimental tissues exposed to botulinum neurotoxin. In each case the protocol was to record spontaneous MEPP's for a baseline period of time, then use potassium at 25 mM to produce a marked increase in MEPP's. Toxin was added and MEPP's were monitored for varying lengths of time. At the end of the experiment, the potassium-induced increase in MEPP's was repeated.

Figures 3 to 8 illustrate some of the most noteworthy findings, as follows:

Figure 3. Control tissues were stable over time. The rate of spontaneous MEPP's was maintained for more than four hours. In addition, potassium depolarization (a, b, c) consistently elevated MEPP rate.

Figure 4. Botulinum neurotoxin type A (1×10^{-8} M) produced blockade of acetylcholine release. Prior to addition of toxin, addition of potassium greatly increase the rate of MEPP's (a). After addition of toxin (b), the rate of MEPP's decayed until they were essentially undetectable. At this point, addition of potassium failed to evoke an increase in MEPP's (c).

Figure 5. The results with serotype A were representative of those obtained with other serotypes known to poison humans (e.g., B and E). However, the experiments yielded a very surprising result. Botulinum neurotoxin type C also blocked transmission. Indeed, it appeared to be somewhat more potent than serotype A. Thus, MEPP rate decreased after addition of toxin (b). Also, the effect of elevated potassium before toxin (a) was virtually abolished after toxin (c).

Figure 6. The paradigm here was identical to that in Figures 4 and 5, but with one important exception. Antibody specific to C was added during the baseline period. In this case, the rate of spontaneous MEPP's before and after toxin (b) did not change. Also, the rate of potassium-induced increases in MEPP's were the same before (a) and after (c) toxin. This demonstrates that

it truly was serotype C that was blocking human neuromuscular transmission.

Figure 7. Although serotype C blocked human transmission, serotype D did not. When added at 1 x 10^{-8} M (b), the toxin did not affect the rate of spontaneous or potassium-induced MEPP's.

Figure 8. This experiment was identical to that in Figure 7, except that a 10-fold higher toxin concentration was used (1 x 10^{-7} M). Still, there was no evidence for blockade of transmission.

The data strongly suggest that the pyramidalis muscle can be used to study toxin action on human neuromuscular transmission. Therefore, human tissues are now being used to gauge the potential effectiveness of toxin antagonists. These include the two classes of drugs described in earlier sections of this report, chelators of zinc and inhibitors of vacuolar ATPase.

B. COMMENT

There are two prominent implications that stem from this work. The first has just been alluded to. The pyramidalis muscle has been identified as a useable preparation for doing human studies. As a result, the findings reported here represent the first systematic analysis of botulinum toxin action on isolated human tissues. Clearly, this work needs to be pursued.

The second major implication relates to botulinum neurotoxin type C. Epidemiology research suggests that humans are resistant to serotypes C and D. This was confirmed for serotype D at the isolated tissue level (Figures 7 and 8). Additional work on ligand binding (unpublished) suggests that human brain tissue may be deficient in its ability to bind the toxin. By contrast, serotype C did block human neuromuscular transmission. This means that the apparent absence of accidental human poisoning must be attributable to some ecological factor (e.g., absence of human exposure) or to a pharmacodynamic factor (e.g., ingested toxin is not well absorbed). As long as the ecological and/or pharmacodynamic barriers exist, poisoning will be infrequent and there will be no need for a vaccine. However, in any situation in which the ecological and/or

pharmacodynamic barriers are overcome, poisoning would be a distinct possibility and the need for a vaccine would be compelling.

TABLE 1

ELECTROPHYSIOLOGICAL CHARACTERISTICS OF HUMAN PYRAMIDALIS MUSCLE

CHARACTERISTIC	MEAN ± SE
RMP (4.0 mM K^+)	-61.6 mV (± 1.7; *n*=17)
RMP (12.5 mM K^+)	-61.4 mV (± 0.7; *n*=90)
Spontaneous Activity (4.0 mM K^+)	0.14 MEPPs sec^{-1} (± 0.03; *n*=11)
Spontaneous Activity (12.5 mM K^+)	1.5 MEPPs sec^{-1} (±0.12; *n*=50)
MEPP Amplitude	2.4mV (± 0.08; *n*=27)
MEPP Duration	3.4 ms (±0.19; *n*=24)
Evoked Activity (25 mM K^+)	14.5 MEPPs sec^{-1} (±2.7; *n*=43)

FIGURE 1

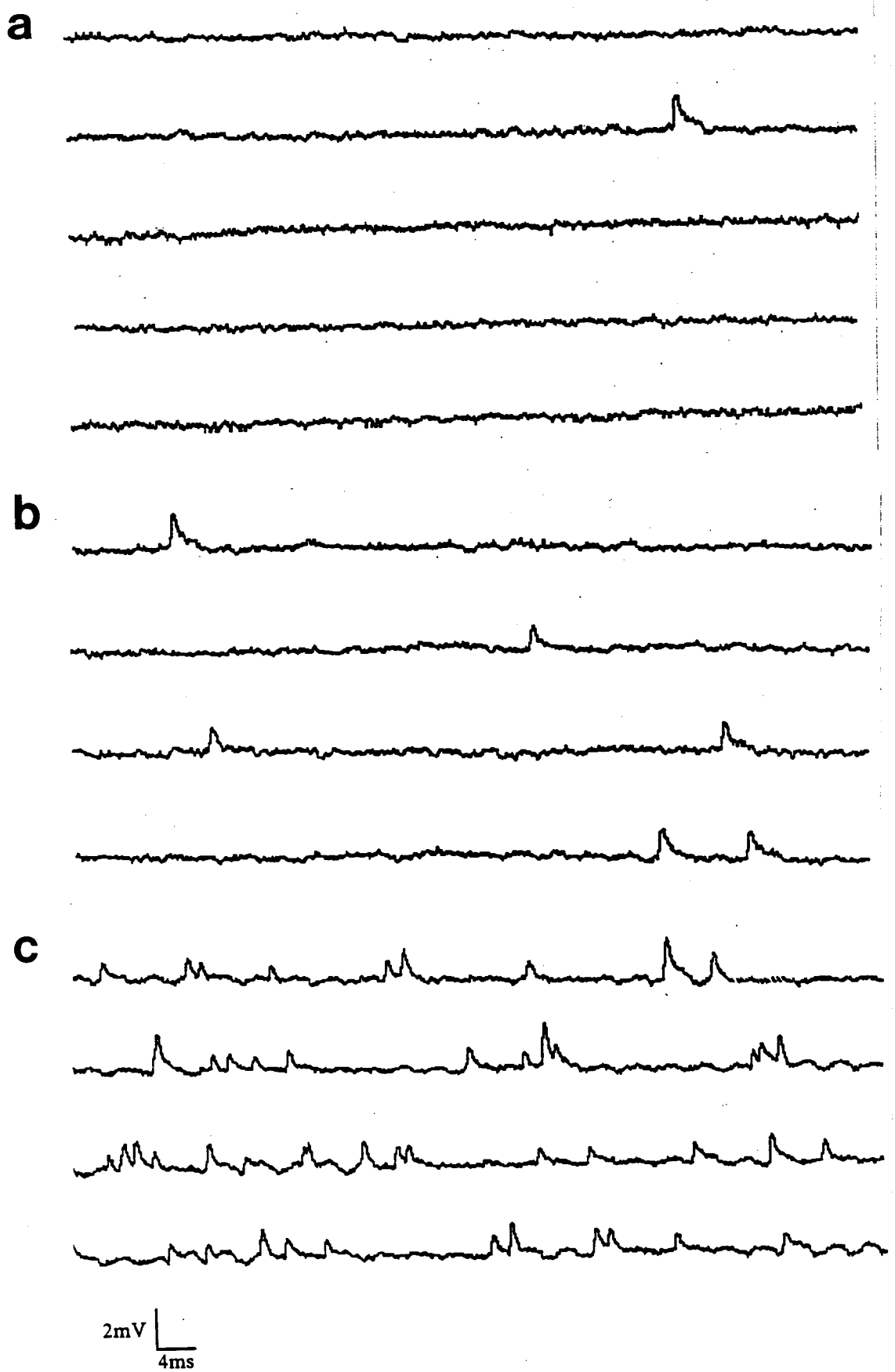


FIGURE 2

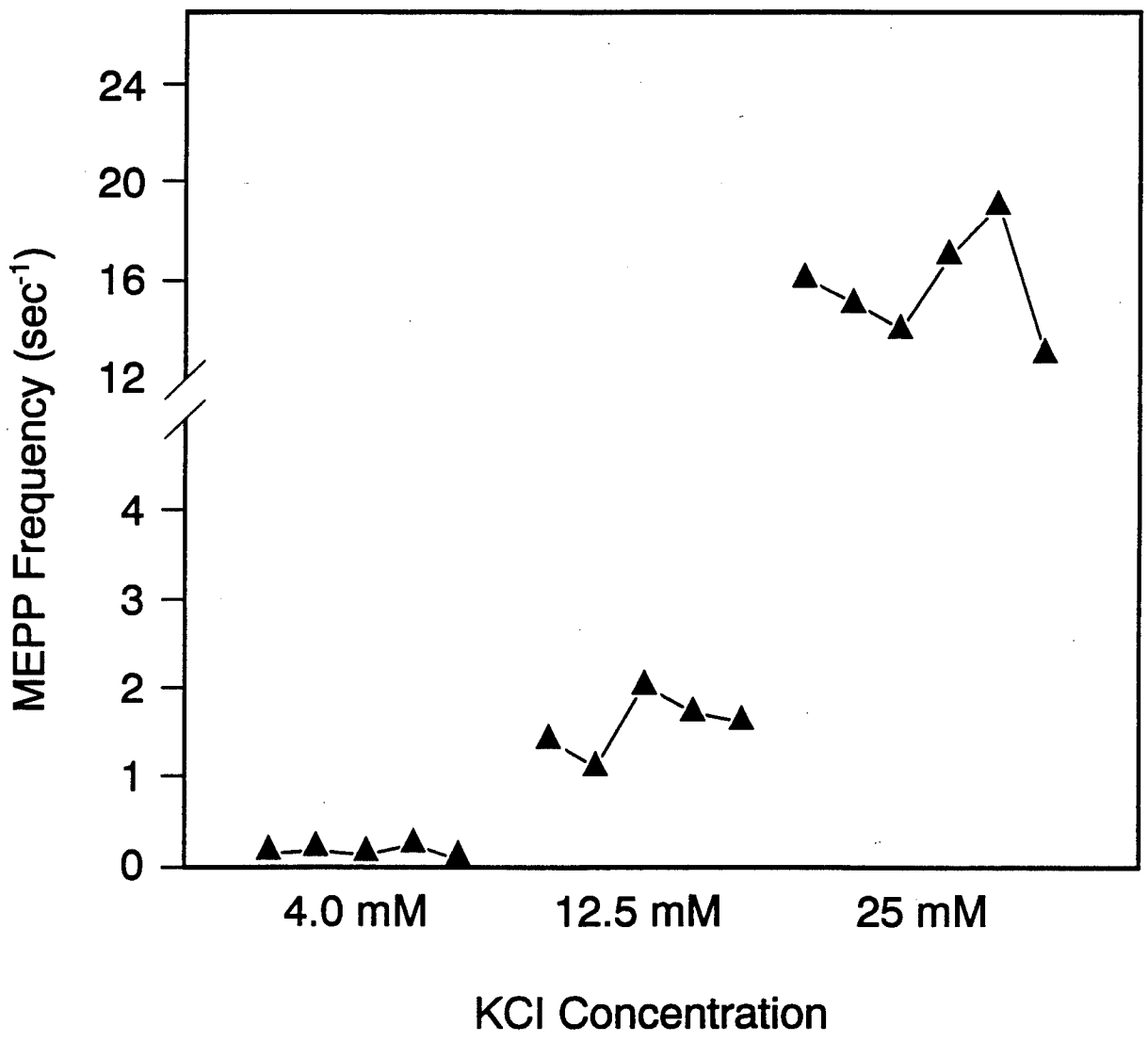

24
20
16
12
4
3
2
1
0
MEPP Frequency (sec-1)
4.0 mM
12.5 mM
25 mM
KCl Concentration

FIGURE 3

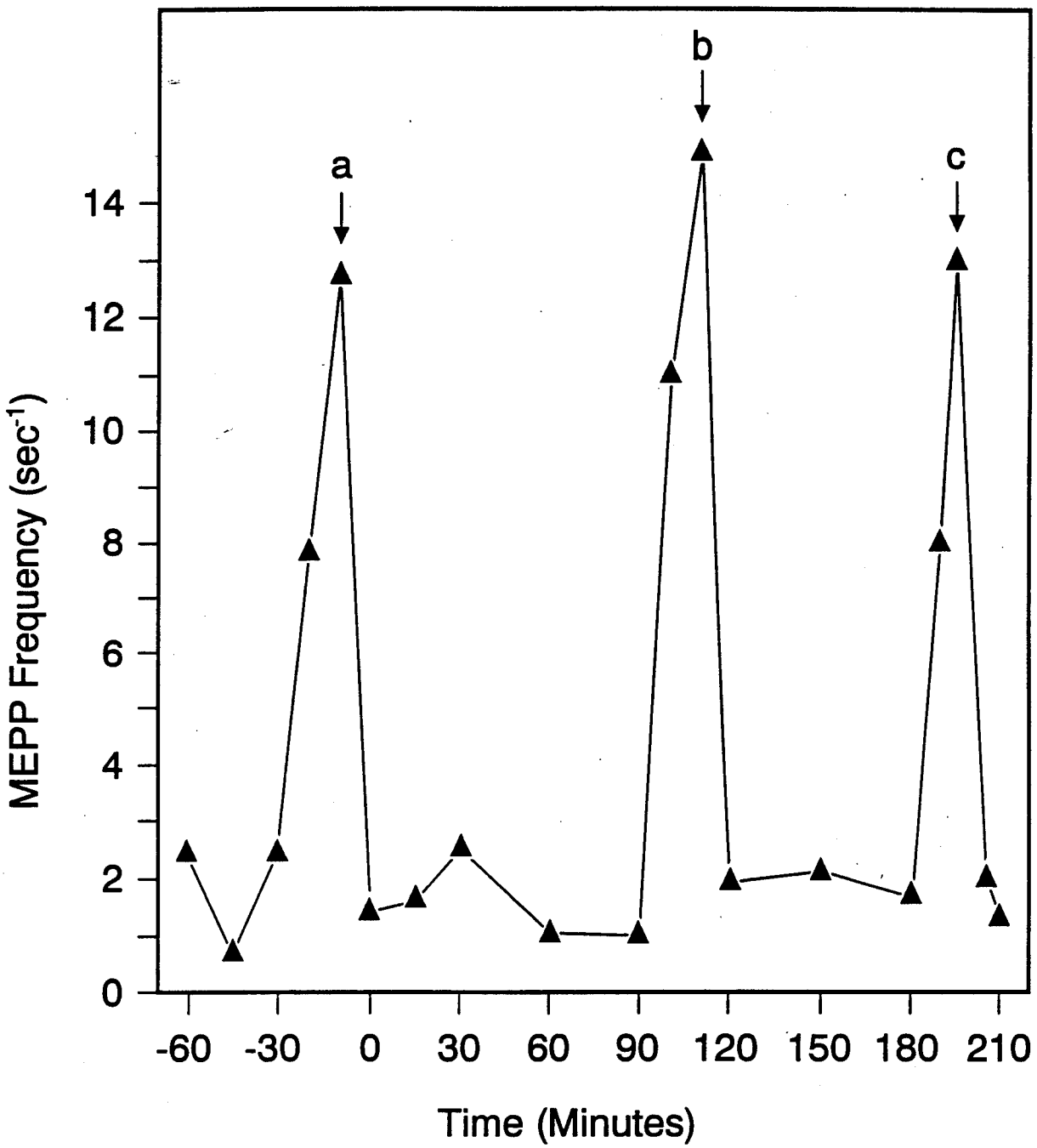

a
b
c
14
12
10
8
6
4
2
0
MEPP Frequency (sec-1)
-60
-30
0
30
60
90
120
150
180
210
Time (Minutes)

FIGURE 4

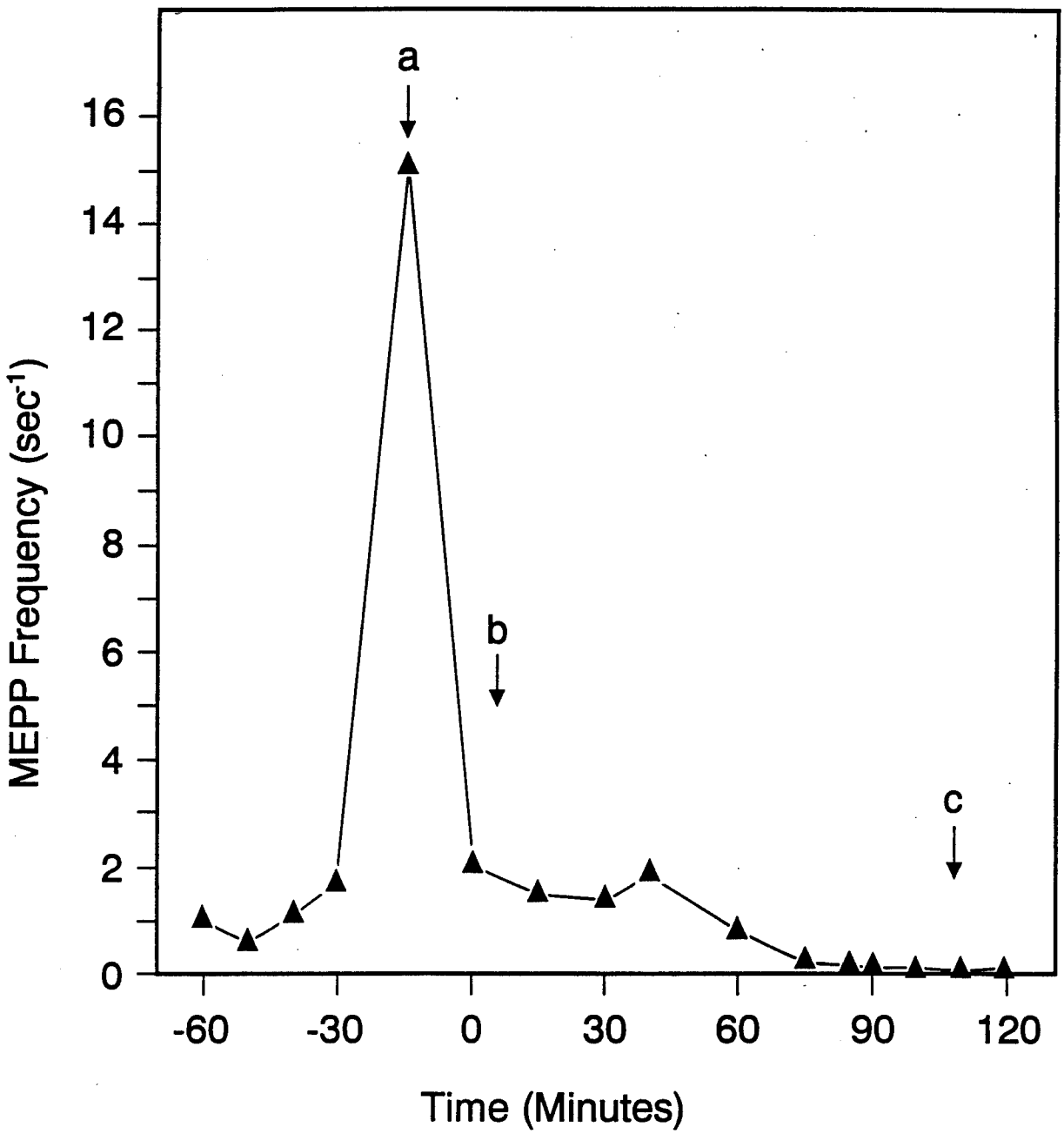

a
b
c
16
14
12
10
8
6
4
2
0
MEPP Frequency (sec-1)
-60
-30
0
30
60
90
120
Time (Minutes)

FIGURE 5

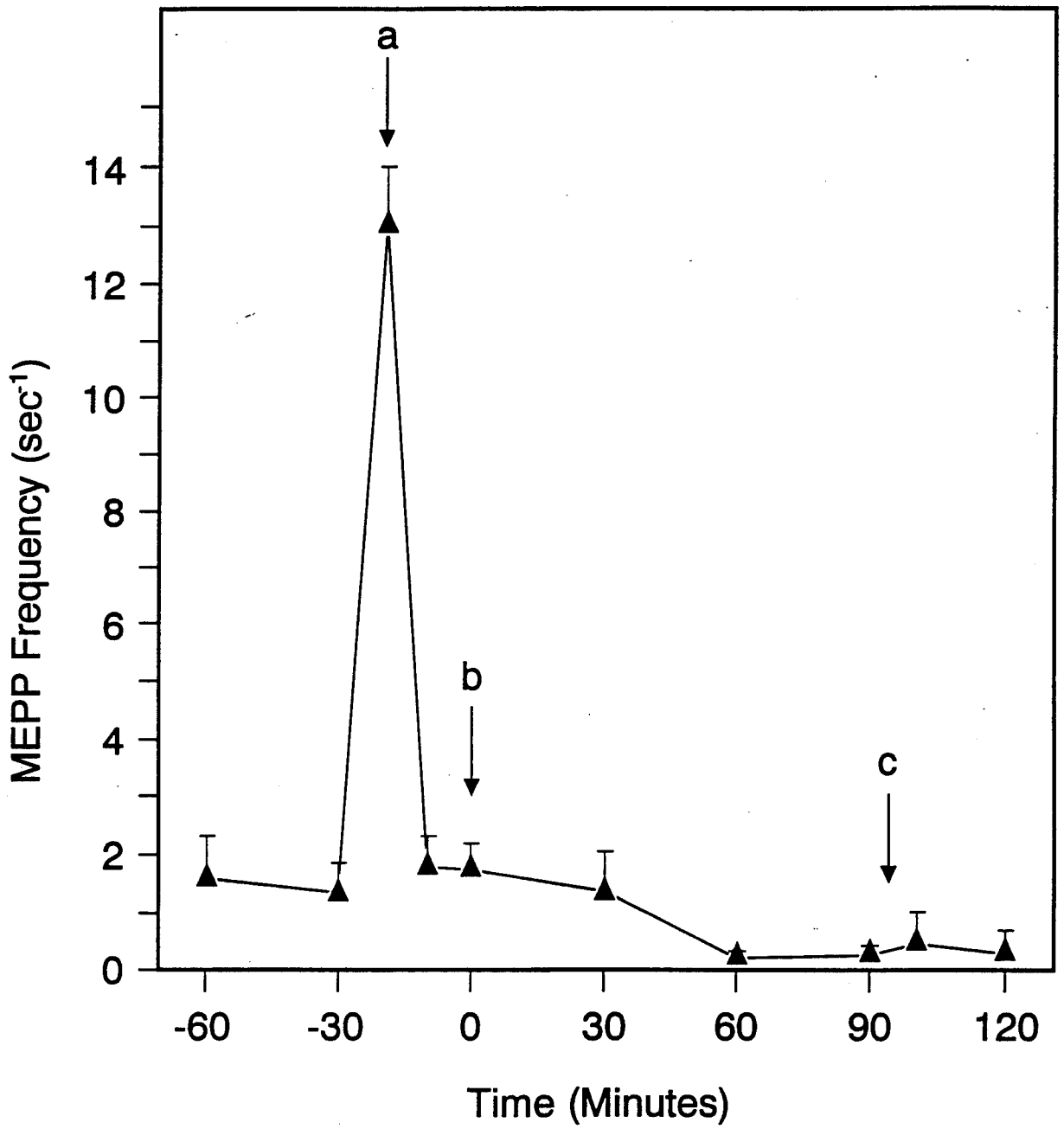

a
b
c
14
12
10
8
6
4
2
0
MEPP Frequency (sec-1)
-60
-30
0
30
60
90
120
Time (Minutes)

FIGURE 6

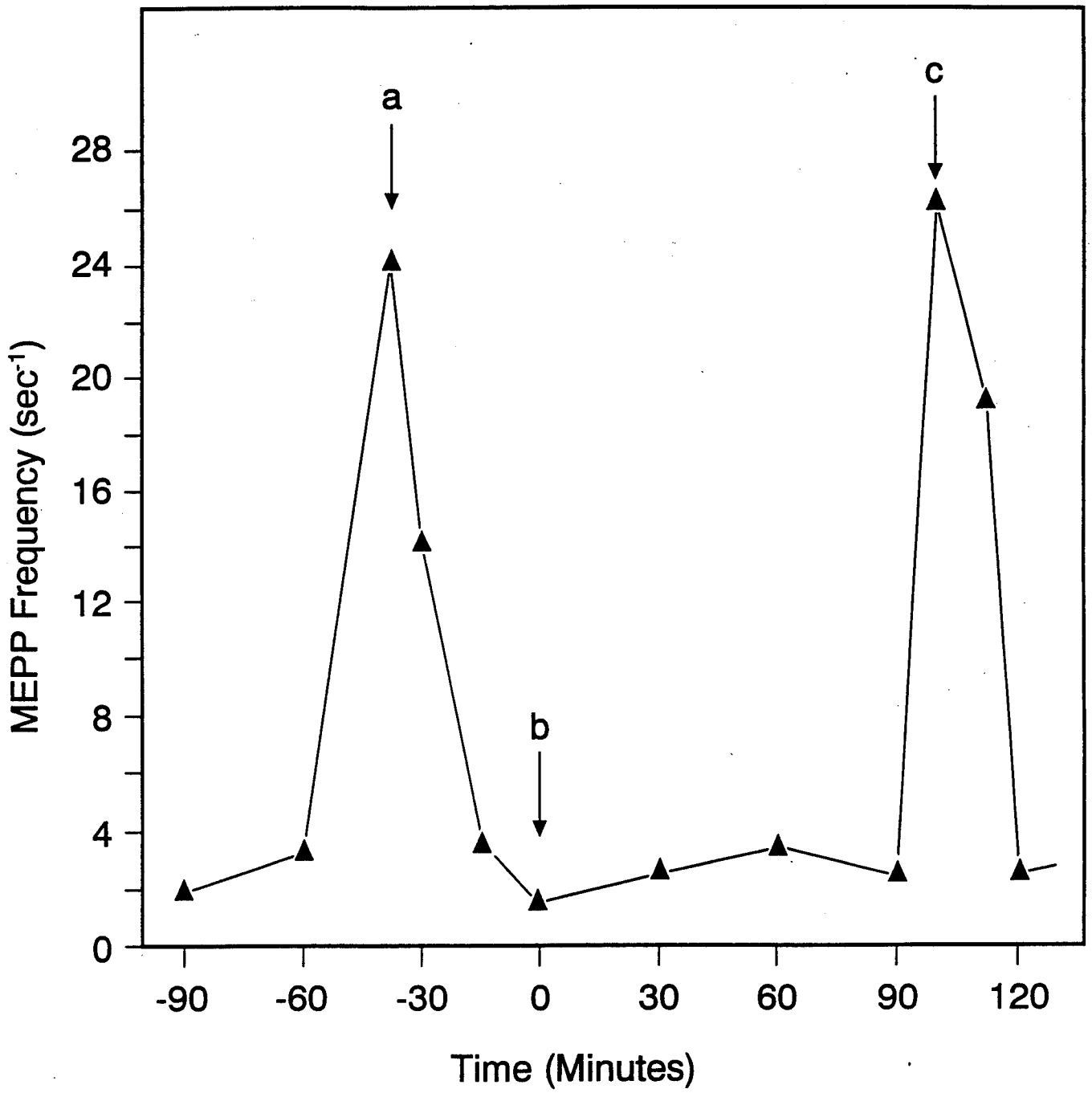

a
b
c
28
24
20
16
12
8
4
0
MEPP Frequency (sec-1)
-90
-60
-30
0
30
60
90
120
Time (Minutes)

FIGURE 7

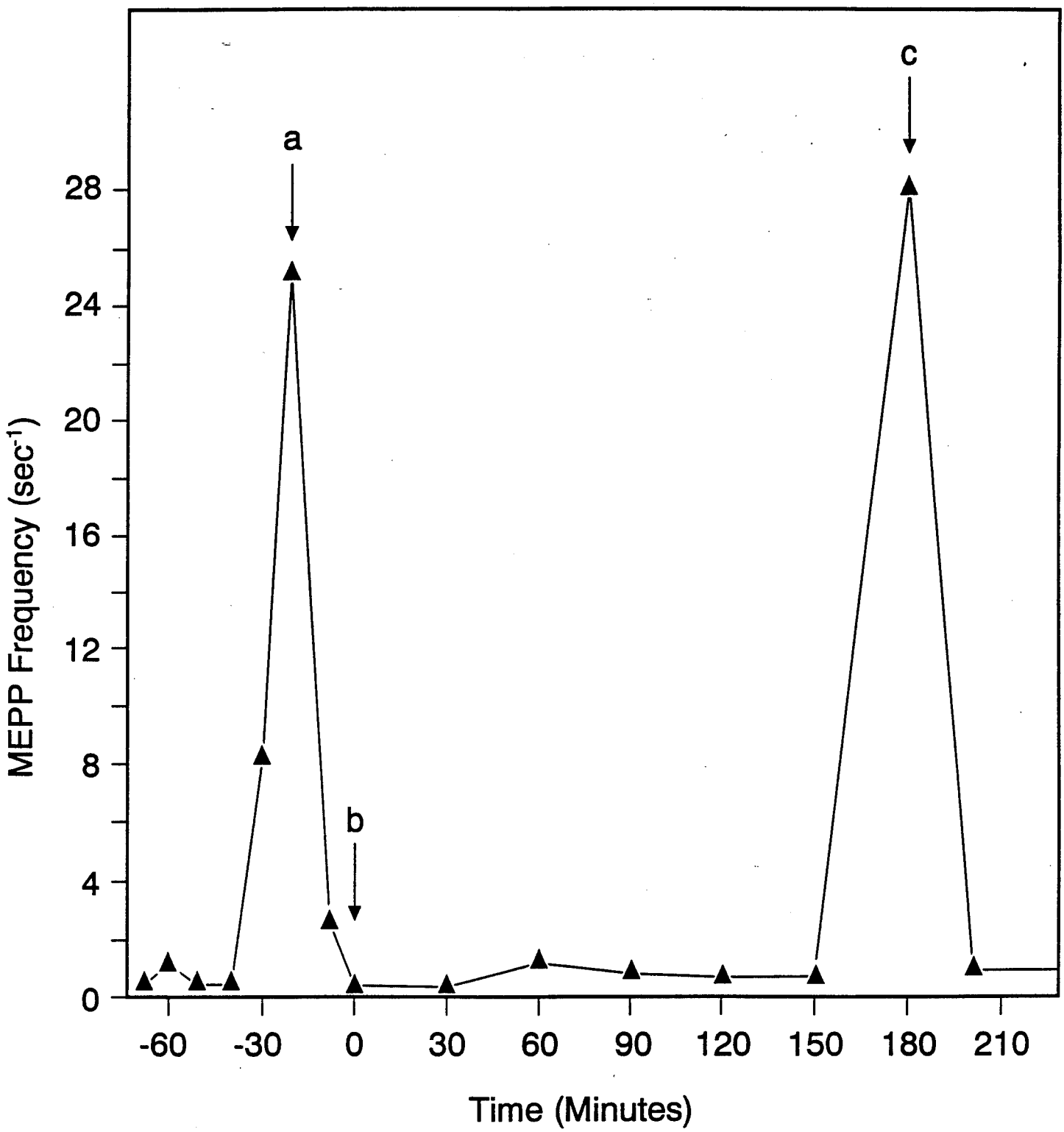
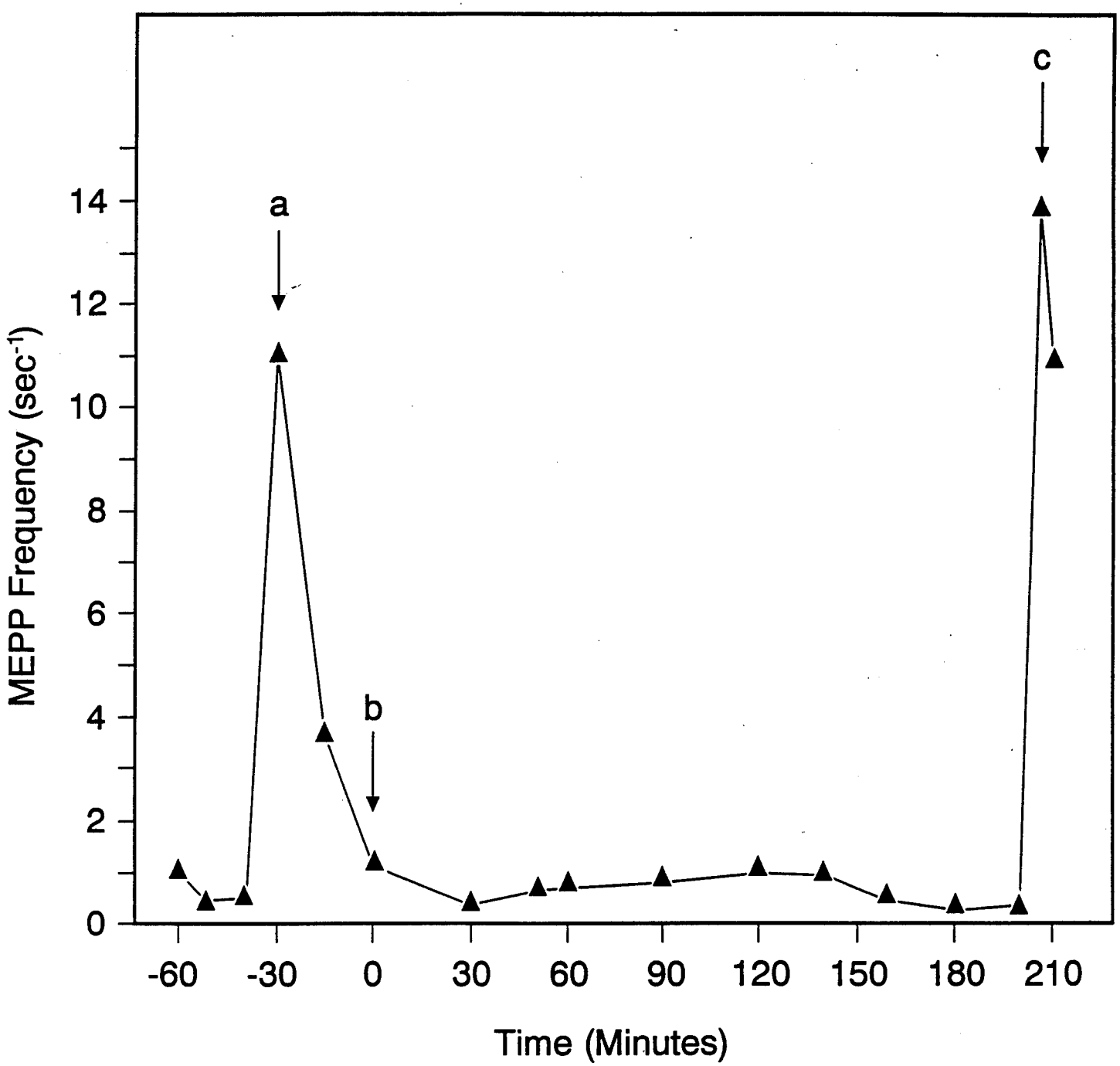

a
b
c
28
24
20
16
12
8
4
0
MEPP Frequency (sec-1)
-60
-30
0
30
60
90
120
150
180
210
Time (Minutes)

FIGURE 8


a
b
c
MEPP Frequency (sec-1)
0
2
4
6
8
10
12
14
-60
-30
0
30
60
90
120
150
180
210
Time (Minutes)

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XIII. PERSONNEL

A. RECEIVING PAY

Nabil Bakry, Ph.D.

Lance Simpson, Ph.D.

Masaru Tanaka, Ph.D.

Ren-de Zhang, M.D., Ph.D.

B. RECEIVING DEGREES

None